JOINT APPENDIX 23

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PATENT

Attorney Docket No.: 086399-001220US-0911148

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 1042
Ian MacLachlan et al.	Examiner: Hirt, Erin E.
Application No.: 14/304,578	Art Unit: 1616
Filed: June 13, 2014	AMENDMENT
For: LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY	
Customer No.: 20350	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

  In response to the Office Action mailed October 9, 2015, please enter the following amendments and remarks.

  Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

  Remarks begin on page 4 of this paper.

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JA00674
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PATENT

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1   1.   (Currently amended) A lipid vesicle formulation comprising:

2   (a) a plurality of lipid vesicles, wherein each lipid vesicle comprises:

3   a cationic lipid;

4   an amphipathic lipid; and

5   a polyethyleneglycol (PEG)-lipid; and

6   (b) messenger RNA (mRNA), wherein at least 70% [[50%]] of the mRNA in the

7   formulation is fully encapsulated in the lipid vesicles.

1   2.   (Previously presented) The lipid vesicle formulation of claim 1, wherein

2   the amphipathic lipid is a phospholipid.

1   3.   (Previously presented) The lipid vesicle formulation of claim 2, wherein

2   the phospholipid is selected from the group consisting of phosphatidylcholine,

3   phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid,

4   palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine,

5   dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine,

6   and dilinoleoylphosphatidylcholine.

1   4.   (Previously presented) The lipid vesicle formulation of claim 1, wherein

2   each lipid vesicle further comprises a sterol.

1   5.   (Previously presented) The lipid vesicle formulation of claim 4, wherein

2   the sterol is cholesterol.

1   6.   (Previously presented) The lipid vesicle formulation of claim 4, wherein

2   the sterol is cholesterol and the amphipathic lipid is a phospholipid.

1   7. (Previously presented) The lipid vesicle formulation of claim 6, wherein

2 the phospholipid is selected from the group consisting of phosphatidylcholine,

3 phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid,

4 palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine,

5 dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine,

6 and dilinoleoylphosphatidylcholine.

1   8. (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 each lipid vesicle is a liposome.

1   9. (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 each lipid vesicle is a lipid-nucleic acid particle.

1   10. (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 each lipid vesicle is about 150 nm or less in diameter.

1   11. (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 the cationic lipid only carries a positive charge at below physiological pH.

1   12. (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 each lipid vesicle is about 100 nm or less in diameter.

1   13. (Canceled)

1   14. (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 at least 80% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

1   15. (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 about 90% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

REMARKS

I.   STATUS OF THE CLAIMS

Upon entry of this amendment, claims 1-12, 14, and 15 are pending in this application and are presented for examination. Claim 1 has been amended. Support is found, for example, in previous claim 13, now canceled. As such, no new matter has been introduced. Based on the following remarks, Applicants respectfully request reconsideration and allowance of the pending claims.

II.   DOUBLE PATENTING REJECTIONS

Claims 1, 9, and 10 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 9, 10, 17, and 19 of U.S. Patent No. 8,058,069. Claims 1-7, 9, and 10 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 4-6, 12, and 21 of U.S. Patent No. 8,283,333. Claims 1 and 9 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 4, 10, and 18 of U.S. Patent No. 7,799,565. Claims 1-7 and 9 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1-6, 8, 10, and 11 of U.S. Patent No. 8,466,122. Claims 1-3 and 9 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 8, 14, and 20 of U.S. Patent No. 8,492,359.

In an earnest effort to expedite prosecution, but without acquiescing on the merits of the present rejections, claim 1 has been amended to include the feature of claim 13. Notably, Applicants point out that claim 13 is not part of the present rejections. Therefore, Applicants respectfully request that the Examiner withdraw the present obviousness-type double patenting rejections.

III.   REJECTION UNDER 35 U.S.C. § 103(a)

Claims 1-15 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Saravolac *et al.* (US Patent No. 6,734,171) and Yoshioka *et al.* (US Patent No. 5,593,622). Applicants respectfully traverse.

In the Office Action, the Examiner alleges that it would have been obvious to one of ordinary skill in the art to make liposomes comprising the same components as presently

claimed because Saravolac *et al.* teaches that it was known in the art to make liposomes and lipid-nucleic acid particles from cationic lipids, PEG-lipids, a sterol, and fusogenic lipids which allow for increased encapsulation efficiencies of over 80%, specifically up to about 86%. *See,* Office Action at page 8.

In response, Applicants respectfully submit herewith a Declaration of Dr. James Heyes under 37 C.F.R. § 1.132 (hereinafter, "Heyes Declaration") to present evidence that the method described in Saravolac *et al.* for preparing lipid particles containing plasmid DNA is ***not*** ***suitable*** for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed.

As explained by Dr. Heyes in his Declaration, he and his colleagues used the method for preparing lipid particles containing plasmid DNA described in Example 1 of Saravolac *et al.* to determine the suitability of this method for formulating mRNA in lipid vesicles. *See,* Heyes Declaration ¶¶ 8-11.

Based on the results of the experiment (*see,* Heyes Declaration ¶¶ 12 & 13), Dr. Heyes states that the method for preparing lipid particles described in Saravolac *et al.* is ***not*** ***suitable*** for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed, wherein at least 70% of the mRNA in the formulation is fully encapsulated in the lipid vesicles. *See,* Heyes Declaration ¶14. Indeed, Dr. Heyes points out that they were only able to achieve up to 53% encapsulation of the mRNA payload following the method of Saravolac *et al.*, despite using the exact dialysis buffer conditions for obtaining "optimum formulations" as described by Saravolac *et al. See, id.* Furthermore, given the high polydispersity indexes of the lipid particles, they were unable to produce particles of reasonable homogeneity using the method described in Saravolac *et al. See, id.* As a result, based on this experiment, Dr. Heyes explains that the method of Saravolac *et al.* produced a population of lipid particles with a heterogeneous size distribution and that encapsulated only about half of the starting mRNA payload. *See, id.* Moreover, based on Tekmira Pharmaceuticals' clinical experience, and the scientific literature, Dr. Heyes notes that the lipid particles produced by the method of Saravolac *et al.* might invoke an unwanted innate immune response upon systemic administration to a human being. *See, id.* Thus, Dr. Heyes concludes that the method described

in Saravolac *et al.* is ***not amenable*** to the production of the population of lipid vesicles claimed in the present application. *See, id.*

For the foregoing reasons, Dr. Heyes submits that there is ***no motivation*** for one of ordinary skill in the art to take the teaching of Saravolac *et al.* and make a lipid vesicle formulation using mRNA and a specific combination of lipid components with any reasonable expectation that at least 70% of the mRNA in the formulation would be successfully encapsulated in the lipid vesicles. *See*, Heyes Declaration ¶15. In fact, Dr. Heyes points out that none of the examples in Saravolac *et al.* discloses or suggests a lipid vesicle formulation of the present invention comprising fully encapsulated mRNA or the desirability of forming more homogeneous particle populations that are more effective at delivering encapsulated nucleic acid molecules such as mRNA to living cells and thus more desirable for *in vivo* and clinical applications. *See, id.* Indeed, the experiment described in the Heyes Declaration clearly shows that the method for preparing lipid particles described in Saravolac *et al.* is ***not suitable*** for formulating the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed. *See, id.*

Applicants assert that the teaching of Yoshioka *et al.* does ***not*** remedy the deficiencies in the disclosure of Saravolac *et al.* In fact, Yoshioka *et al.* ***fails*** to provide any teaching whatsoever with regard to mRNA or the successful encapsulation and delivery thereof. Indeed, the Examiner merely relies on Yoshioka *et al.* for teaching the selection of cholesterol and certain phospholipids for inclusion in the lipid particles of Saravolac *et al.* *See,* Office Action at pages 7-8.

In view of the foregoing, Applicants assert that the cited references, whether alone or in combination, do ***not*** teach or suggest each of the features recited in the instant claims and thus fail to support a legal conclusion of obviousness. Indeed, ***none*** of these references discloses or suggests a lipid vesicle formulation of the present invention comprising a plurality of lipid vesicles and mRNA, wherein at least 70% of the mRNA in the formulation is fully encapsulated in the lipid vesicles. To the contrary, Applicants have provided sufficient objective evidence in the Heyes Declaration to demonstrate that the method described in Saravolac *et al.* for preparing lipid particles is simply ***not suitable*** for producing the population of lipid vesicles with an

Appl. No. 14/304,578   <u>PATENT</u>
Amdt. dated December 14, 2015
Reply to Office Action of October 9, 2015

mRNA encapsulation efficiency as presently claimed. The teaching of Yoshioka *et al.* does ___not___

remedy this deficiency in the method of Saravolac *et al.* Accordingly, Applicants respectfully

request that the Examiner withdraw the present rejection under 35 U.S.C. § 103(a).

<div align="center"><u>CONCLUSION</u></div>

In view of the foregoing, Applicants believe all claims now pending in this

application are in condition for allowance. The issuance of a formal Notice of Allowance at an

early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of

this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,

/Joe C. Hao/

Joe C. Hao
Reg. No. 55,246

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Attachments
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

    Ian MacLachlan et al.

Application No.: 14/304,578

Filed: June 13, 2014

For: LIPID COMPOSITIONS FOR
NUCLEIC ACID DELIVERY

Customer No.: 20350

Confirmation No. 1042

Examiner: Hirt, Erin E.

Art Unit: 1616

<u>DECLARATION UNDER 37 C.F.R. § 1.132</u>

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

    I, James Heyes, Ph.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

    1.   All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

    2.   I hold a Ph.D. (2001) in Medicinal Chemistry from the Institute of Cancer Research (Surrey, UK).   I am presently the Director of Formulation Chemistry at Arbutus Biopharma Corporation (Burnaby, Canada), formerly known as Tekmira Pharmaceuticals Corporation. The assignee of the above-referenced application, Protiva Biotherapeutics Inc., is a wholly-owned subsidiary of Arbutus Biopharma.

    3.   My expertise lies in the development of lipid particle formulations and the design of novel compounds as components of lipid particles. A copy of my *Curriculum Vitae* is of record.

4.   I have reviewed the above-referenced patent application, and I am familiar with the contents therein. I have also reviewed the contents of the Office Action dated October 9, 2015.

5.   The present invention is directed to a lipid vesicle formulation comprising: (a) a plurality of lipid vesicles, wherein each lipid vesicle comprises: a cationic lipid; an amphipathic lipid; and a polyethyleneglycol (PEG)-lipid; and (b) messenger RNA (mRNA), wherein at least 70% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

6.   In the Office Action, the Examiner relies on Saravolac *et al.* (US Patent No. 6,734,171) in alleging that it would have been obvious to one of ordinary skill in the art to make liposomes comprising the same components as presently claimed because Saravolac *et al.* teaches that it was known in the art to make liposomes and lipid-nucleic acid particles from cationic lipids, PEG-lipids, a sterol, and fusogenic lipids which allow for increased encapsulation efficiencies of over 80%, specifically up to about 86%. *See,* Office Action at page 8.

7.   I submit this Declaration to present evidence that the method described in Saravolac *et al.* for preparing lipid particles containing plasmid DNA is not suitable for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed that is desirable for *in vivo* and clinical applications.

8.   My colleagues and I used the method for preparing lipid particles containing plasmid DNA described in Example 1 of Saravolac *et al.* to determine the suitability of this method for formulating mRNA in lipid vesicles.

9.   The method described in Saravolac *et al.* was followed, except for a single, minor modification in preparing the lipid stock solutions that, to the best of my knowledge and belief, is unlikely to affect the outcome of the experiment described herein. In particular, lipids were dissolved in 100% chloroform, instead of absolute ethanol, 2:1 chloroform:methanol, or 9:1 benzene:methanol as described in Saravolac *et al.* *See,* col. 21, lines 32-34. Since this solvent is evaporated after aliquoting the lipids, and prior to formulating

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the particles, to the best of my knowledge and belief, the use of 100% chloroform has no effect on the process, as the chloroform fully dissolves the lipids and is evaporated afterward.

10. In addition, Saravolac *et al.* notes that "optimum formulations are obtained with 150 mM NaPO$_4$, pH 7.4 with 150 to 175 mM NaCl" in the dialysis buffer. *See,* col. 21, lines 55-56. We therefore performed the experiment with both the lower (150 mM) and upper (175 mM) concentration of NaCl in the dialysis buffer, and the results of both formulations are described below.

11. The experiment was performed as follows:

Preparation of Lipid Particles: DOPE, DODAC, and PEG$_{2000}$-CerC8 individual stock solutions were prepared at 100 mg/mL in chloroform. Aliquots were combined to give a molar ratio of DOPE:DODAC:PEG-CerC8 (42.5:42.5:15 mol%). These were prepared in duplicate, and tubes placed under a stream of N$_2$ to evaporate off the solvent. Finally, they were exposed to vacuum to remove any trace amounts of the chloroform remaining. To the dried lipid film was added 100 µL of 1 M octyl glucopyranoside (OGP), 200 µL of 1.0 mg/mL luciferase mRNA, and 700 µL of PBS buffer containing either 150 mM or 175 mM NaCl. The tubes were then vigorously vortexed to solubilize the lipid films. Upon complete solubilization, the mixtures were transferred to dialysis tubing (3 mL Slide-A-Lyzers with MWCO of 10,000) and the first sample was dialyzed against 150 mM NaPO$_4$, 150 mM NaCl, pH 7.4, while the other was dialyzed against 150 mM NaPO$_4$, 175 mM NaCl, pH 7.4. Samples were dialyzed against 1.5 L of dialysis buffer over a 24 hour period with 2 changes of buffer over this time. Upon completion of dialysis, the samples were removed from the dialysis bags and analyzed for size and percent encapsulation.

12. Upon completion of dialysis, the formulations were assessed. The results of the experiment are summarized in Table 1. Three formulation parameters were measured, each in duplicate:

- % Encapsulation of mRNA (*i.e.,* the amount of mRNA that has been successfully encapsulated in the particle);

- Particle size; and
- Polydispersity (*i.e.*, a measure of the heterogeneity of sizes of particles in a mixture).

Table 1. Size of particles and encapsulation of mRNA following dialysis

Dialysis Buffer	Replicate	Diameter (nm)	Polydispersity index (PDI)	% Encapsulation
150 mM NaPO$_4$, 150 mM NaCl, pH 7.4	1	84	0.34	50%
	2	86	0.35	53%
150 mM NaPO$_4$, 175 mM NaCl, pH 7.4	1	75	0.32	45%
	2	76	0.26	47%

13.   Similar results were obtained for both formulations containing different NaCl concentrations. In particular, a number of clear deficiencies in the lipid particles prepared by the method described in Saravolac *et al.* were observed for both formulations. First, only about half of the mRNA payload was successfully encapsulated into lipid particles. Second, the polydispersity index (PDI) was high (0.26-0.35). This measurement reflects how homogeneous a formulation is from a size perspective, with a lower number (*i.e.*, PDI ~0.1 or less) being desirable and reflecting a more homogeneous particle population. A PDI value around 0.3 indicates a broad range of particle sizes in the mixture, which is extremely undesirable in a lipid particle delivery system. The substantial amount of unencapsulated mRNA and the high polydispersity index together or individually increase the likelihood of an unwanted immune response upon *in vivo* administration of the lipid particles.

14.   This experiment demonstrates that the method for preparing lipid particles described in Saravolac *et al.* is not suitable for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed, wherein at least 70% of the mRNA in the formulation is fully encapsulated in the lipid vesicles. Indeed, we were only able to achieve up to 53% encapsulation of the mRNA payload following the method of Saravolac *et al.*, despite using the exact dialysis buffer conditions for obtaining "optimum formulations" as described by Saravolac *et al.* Furthermore, given the high polydispersity indexes of the lipid particles, we

were unable to produce particles of reasonable homogeneity using the method described in Saravolac *et al.* Thus, based on the experiment reported herein, the method of Saravolac *et al.* produced a population of lipid particles with a heterogeneous size distribution and that encapsulated only about half of the starting mRNA payload.   Based on Tekmira Pharmaceuticals' clinical experience, and the scientific literature, the lipid particles produced by the method of Saravolac *et al.* reported herein might invoke an unwanted innate immune response upon systemic administration to a human being.   Based on this experiment, I conclude that the method described in Saravolac *et al.* is not amenable to the production of the population of lipid vesicles claimed in the present application.

15.   For the foregoing reasons, I submit that there is no motivation for one of ordinary skill in the art to take the teaching of Saravolac *et al.* and make a lipid vesicle formulation using mRNA and a specific combination of lipid components with any reasonable expectation that at least 70% of the mRNA in the formulation would be successfully encapsulated in the lipid vesicles.   In fact, none of the examples in Saravolac *et al.* discloses or suggests a lipid vesicle formulation of the present invention comprising fully encapsulated mRNA or the desirability of forming more homogeneous particle populations that are more effective at delivering encapsulated nucleic acid molecules such as mRNA to living cells and thus more desirable for *in vivo* and clinical applications.   Indeed, our experiment clearly shows that the method for preparing lipid particles described in Saravolac *et al.* is not suitable for formulating the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed.

16.   The declarant has nothing further to say.

Dec 7ᵗʰ, 2015
_____
Date

_____
James Heyes, Ph.D.

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JOINT APPENDIX 24

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/304,578	06/13/2014	Ian MacLachlan	86399-001220US-911148	1042

20350 7590 04/15/2016 KILPATRICK TOWNSEND & STOCKTON LLP TWO EMBARCADERO CENTER EIGHTH FLOOR SAN FRANCISCO, CA 94111-3834	**EXAMINER** HIRT, ERIN E

ART UNIT	PAPER NUMBER
1616	

NOTIFICATION DATE	DELIVERY MODE
04/15/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipefiling@kilpatricktownsend.com
jlhice@kilpatrick.foundationip.com

PTOL-90A (Rev. 04/07)

Office Action Summary	Application No. 14/304,578	Applicant(s) MACLACHLAN ET AL.		
	Examiner ERIN HIRT	Art Unit 1616	AIA (First Inventor to File) Status No	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE *3* MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☒ Responsive to communication(s) filed on *12/14/15*.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a) ☒ This action is **FINAL**.   2b) ☐ This action is non-final.

3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☒ Claim(s) *1-12,14 and 15* is/are pending in the application.
   5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☒ Claim(s) *1-12,14 and 15* is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined <u>allowable</u>, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10) ☐ The specification is objected to by the Examiner.

11) ☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.
    Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
    Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
    Certified copies:
    a)☐ All   b)☐ Some** c)☐ None of the:
        1. ☐ Certified copies of the priority documents have been received.
        2. ☐ Certified copies of the priority documents have been received in Application No. _____.
        3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.

4) ☐ Other: _____.

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Art Unit: 1616

The present application is being examined under the pre-AIA first to invent

provisions.

DETAILED ACTION

Status of Action

The examiner acknowledges receipt of Amendments/Remarks filed on 12/14/15.

Currently claims 1-12, 14-15 are pending in this application. Claim 13 was canceled.

Status of Claims

Accordingly, claims 1-12 and 14-15 are presented for examination on the merits

for patentability. Rejection(s) not reiterated from the previous Office Action are hereby

withdrawn. The following rejections are either reiterated or newly applied. They

constitute the complete set of rejections presently being applied to the instant

application.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all

obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148

USPQ 459 (1966), that are applied for establishing a background for determining

obviousness under 35 U.S.C. 103(a) are summarized as follows:

1.   Determining the scope and contents of the prior art.
2.   Ascertaining the differences between the prior art and the claims at issue.

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Art Unit: 1616

    3.   Resolving the level of ordinary skill in the pertinent art.

    4.   Considering objective evidence present in the application indicating
           obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of
the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of
the various claims was commonly owned at the time any inventions covered therein
were made absent any evidence to the contrary. Applicant is advised of the obligation
under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was
not commonly owned at the time a later invention was made in order for the examiner to
consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g)
prior art under 35 U.S.C. 103(a).

Claims 1-12, 14-15 are rejected under 35 U.S.C. 103(a) as being unpatentable
over Saravolac et al. (US6734171) and Yoshioka et al. (US5593622).

Applicant's claim:

A lipid vesicle comprising:
a messenger RNA (mRNA);
a cationic lipid;
an amphipathic lipid; and
a polyethyleneglycol (PEG)-lipid; and mRNA, wherein at least 50% of the mRNA in the
formulation is fully encapsulated in the lipid vesicles.

Determination of the scope and content of the prior art

(MPEP 2141.01)

Regarding claims 1-3 and 6-7, Saravolac teaches methods of encapsulating

mRNA/nucleic acids in lipid bilayers to form liposomes and lipid-nucleic acid particles

(see entire document; Col. 2, ln. 23-44; Col. 12, ln. 8-28). **Further regarding claims 1-**

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Art Unit: 1616

3 and 6-7, Saravolac teaches lipid vesicles comprising a cationic lipid (i.e. DODAC), an

amphipathic/fusogenic lipid, specifically a phospholipid such as DOPE

(dioleylphosphatidylethanolamine) or lysophosphatidylcholine or

lysophosphatidylethanolamine, preferably a PEG-lipid, and mRNA/plasmid, specifically

wherein at least 70% of the mRNA is fully encapsulated in the lipid vesicles (see entire

document; Col 2, ln. 45-54; Col. 8, ln. 33-Col. 9, ln. 25; Col. 10, ln. 12-Col. 11, ln. 8; Col.

11, ln. 26-35; Col. 12, ln. 29-Col. 14, ln. 33; Col. 14, ln. 60-Col. 15, ln. 12; Figure 8).

Regarding claim 4, Saravolac teaches wherein the lipid vesicles can further

comprise a sterol (see entire document; Col. 8, ln. 55-57).

Regarding claims 10 and 12, Saravolac teaches wherein the diameter of the

lipid vesicles are about 100 nm or less in diameter which reads upon the instantly

claimed 150 nm or less and 100 nm or less (see entire document; e.g. Claim 15;

Examples).

Regarding claims 8-9, Saravolac teaches wherein the lipid vesicle is either a

liposome or a lipid-nucleic acid particle (see entire document; Col. 12, ln. 8-28).

Regarding claims 1, 14-15, Saravolac teaches wherein the encapsulation

efficiency of the mRNA in the vesicles is about 80% and can even approach 90% which

reads upon the instantly claimed at least 70%, more specifically at least 80%, and about

90% (see entire document; Col 2, ln. 45-54; see Fig. 8).

Ascertainment of the difference between prior art and the claims

(MPEP 2141.02)

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Regarding claim 5, Saravolac does not specifically teach wherein the sterol is

cholesterol. However, this deficiency in Saravolac is addressed by Yoshioka.

Yoshioka teaches incorporating cholesterol into liposomes as membrane

stabilizing agents (see entire document; Col. 5, ln. 20-23).

Regarding claims 3 and 7, Saravolac does not specifically teach an example

wherein the fusogenic lipid is other than DOPE. However, Saravolac teaches that

lysolipids, specifically the instantly claimed lysophosphatidylcholine or

lysophosphatidylethanolamine are useful as lysolipids/fusogenic lipids for forming the

instantly claimed lipid vesicles (see entire document; Col. 13, ln. 22-Col. 14, ln. 48).

<div align="center">

Finding of prima facie obviousness

Rationale and Motivation (MPEP 2142-2143)

</div>

It would have been obvious to one of ordinary skill in the art at the time of the

instant invention to make liposomes having a cationic lipid, a PEG-lipid, a sterol, and a

phospholipid, specifically lysophosphatidylcholine or lysophosphatidylethanolamine as

instantly claimed because Saravolac teaches that it was known in the art to make

liposomes and lipid-nucleic acid particles from cationic lipids, PEG-lipids, a sterol, and

fusogenic lipids such as the instantly claimed lysophosphatidylcholine or

lysophosphatidylethanolamine which allow for increased encapsulation efficiencies of

over 80%, specifically up to about 86%.

Regarding the selection of cholesterol as the sterol in the liposomes and lipid-

nucleic acid particles, it would have been obvious to select cholesterol as the sterol for

inclusion in the liposome/lipid-nucleic acid particles of Saralovac because Yoshioka

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teaches that the addition of cholesterol helps to stabilize the membrane of the

liposomes.

Response to Arguments/Remarks

Applicant's amendments to the claims have prompted the revised grounds of

rejection presented herein. Applicant's amendments to the claims have also rendered

the previous double patenting rejections moot, and these rejections were withdrawn by

the examiner. Applicant's arguments insofar as they pertain to the revised grounds of

rejection are presented herein.

Applicants argue that argue that their declaration by Dr. Heyes presents

evidence that the method described in Saravolac is not suitable for producing lipid

vesicles with mRNA encapsulation efficiencies of at least 70% as is instantly claimed.

The examiner is not swayed by this declaration because applicants did not completely

follow the method presented by Saravolac in their experiments. Furthermore, Saravolac

themselves provide evidence that the encapsulation efficiency of their method allows for

particles which contain the all of the same components as are instantly claimed. As

such, Saravolac provided evidence that their method does produce lipid vesicles which

comprise greater that 70% of nucleic acids and it is well known in the art that mRNA is

formed of nucleic acids. The examiner is confused as to why applicants did not

absolutely follow the procedure of Saravolac in their experiments because Saravolac

used ethanol to dissolve their nucleic acid and applicant's own method as described in

their specification also uses ethanol to solubilize their lipids/form their lipid stock

solutions. Therefore, it is unclear to the examiner why Dr. Heyes then seemed to

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arbitrarily pick chloroform since it wasn't used in their own method and wasn't used in

Saravolac to solubilize/form their lipid stock solutions.

Applicant's then argue that there is no motivation for one of ordinary skill in the

art to take the teachings of Saravolac to make the instantly claimed lipid vesicles with

any reasonable expectation of success. The examiner respectfully disagrees because

Saravolac teaches lipid vesicles which comprise all of the same components which can

encapsulate nucleic acids, which obviously includes mRNA because mRNA is made of

nucleic acids, at efficiencies which are reported to be as high as 86% as is reported in

Figure 8. Saravolac merely does not teach wherein the sterol that can be incorporated

into their lipid vesicles is cholesterol. However, cholesterol was known in the art to

stabilize lipid membranes as is taught by Yoshioka, and because of this stabilization it

would have been obvious to one of ordinary skill in the art to select cholesterol as the

sterol for use in the lipid vesicles of Saravolac because it stabilizes the lipid

membrane(s). Applicants have further provided a declaration which has been

addressed below.

Declaration under 1.132

Dr. James Heyes provided a declaration under 1.132, filed 12/14/15 and dated

12/7/15. The Declaration meets the formal requirements. In the most relevant part, the

Declaration demonstrates that at a buffer concentration of 150 mM NaPO4 and 150 mM

NaCl and 150 mM NaPO4 and 175 mM NaCl lipid vesicles of ~75-86 nm and

polydispersity index of ~0.32 are formed and that these particles have encapsulated

about 50% of the nucleic acids/mRNA. A Declaration is due full consideration and

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weight for all that it discloses. Declarations are reviewed for the following

considerations: 1) whether the Declaration presents a nexus such as a side-by-side or

single-variable comparison (In re Huang, 40 USPQ2d 1685, 1689 (Fed. Cir. 1996)), 2)

whether the Declaration presents a comparison to the closest art, 3) whether the

Declaration is commensurate in scope with the scope of the claims (In re Kulling, 14

USPQ2d 1056, 1058 (Fed. Cir. 1990)), 4) whether the Declaration shows a difference in

kind rather than merely a difference in degree (In re Waymouth, 182 USPQ 290, 293

(C.C.P.A. 1974)), and 5) whether the prima facie case is sufficiently strong that

allegedly superior results are insufficient to overcome the case for obviousness (Pfizer

Inc. v. Apotex, Inc., 82 USPQ2d 1321, 1339 (Fed. Cir. 2007)). The relevant criterion

here is No. 5, whether the prima facie case is sufficiently strong that allegedly superior

results are insufficient to overcome the case for obviousness. The examiner has

carefully reviewed the Declaration, including the data presented in the Declaration. The

data shows that the disclosed method is capable of encapsulating mRNA with an

efficiency of about 50% and a PDI of about 0.32. However, the examiner respectfully

points out that the rejection of the instant claims does not make any reference to the

polydispersity index of their lipid vesicles, and as such applicants are arguing feature

which have not been claimed. Furthermore, it is unclear to the examiner what the

difference is in the procedures/processes between the data in the instant declaration

and Saravolac which accounts for the differences in the encapsulation efficiencies. Dr.

Heyes further argues that there is no motivation for one of ordinary skill in the art at the

time of the instant invention to take the teaching of Saravolac to make an mRNA

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containing lipid vesicles with any expectation that at least 70% of the mRNA would be

successfully encapsulated. The examiner respectfully disagrees and points applicant's

to these arguments which were addressed above as this appears to be a repeat of the

arguments stated in their arguments/remarks.

In light of the forgoing discussion, the Examiner concludes that the subject matter

defined by the above claims would have been obvious to one of ordinary skill in the art

within the meaning of 35 USC 103(a). From the teachings of the references, it is

apparent that one of ordinary skill in the art would have had a reasonable expectation of

success in producing the claimed invention.

Conclusion

Claims 1-15 are rejected.

Applicant's amendment necessitated the new ground(s) of rejection presented in

this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP

§ 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37

CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE

MONTHS from the mailing date of this action. In the event a first reply is filed within

TWO MONTHS of the mailing date of this final action and the advisory action is not

mailed until after the end of the THREE-MONTH shortened statutory period, then the

shortened statutory period will expire on the date the advisory action is mailed, and any

extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of

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the advisory action. In no event, however, will the statutory period for reply expire later

than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the

examiner should be directed to ERIN HIRT whose telephone number is (571)270-1077.

The examiner can normally be reached on Monday through Friday 9:00am to 6:30pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's

supervisor, Sue X. Liu can be reached on 571-272-5539. The fax phone number for the

organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the

Patent Application Information Retrieval (PAIR) system. Status information for

published applications may be obtained from either Private PAIR or Public PAIR.

Status information for unpublished applications is available through Private PAIR only.

For more information about the PAIR system, see http://pair-direct.uspto.gov. Should

you have questions on access to the Private PAIR system, contact the Electronic

Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a

USPTO Customer Service Representative or access to the automated information

system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Erin Hirt
Examiner, Art Unit 1616

/Mina Haghighatian/
Primary Examiner, Art Unit 1616

JOINT APPENDIX 25

<u>PATENT</u>
Attorney Docket No.: 086399-001220US-0911148

KILPATRICK TOWNSEND & STOCKTON LLP

By: _____ /Judith Cotham/ _____
         Judith Cotham

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 1042
Ian MacLachlan et al.	Examiner: Hirt, Erin E.
Application No.: 14/304,578	Art Unit: 1616
Filed: June 13, 2014	<u>AMENDMENT</u>
For: LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY	
Customer No.: 20350	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the Final Office Action mailed April 15, 2016, please enter the following amendments and remarks. A Request for Continued Examination (RCE) accompanies the present response.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks begin on page 4 of this paper.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1   1.   (Previously presented) A lipid vesicle formulation comprising:

2   (a) a plurality of lipid vesicles, wherein each lipid vesicle comprises:

3   a cationic lipid;

4   an amphipathic lipid; and

5   a polyethyleneglycol (PEG)-lipid; and

6   (b) messenger RNA (mRNA), wherein at least 70% of the mRNA in the

7 formulation is fully encapsulated in the lipid vesicles.

1   2.   (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 the amphipathic lipid is a phospholipid.

1   3.   (Previously presented) The lipid vesicle formulation of claim 2, wherein

2 the phospholipid is selected from the group consisting of phosphatidylcholine,

3 phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid,

4 palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine,

5 dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine,

6 and dilinoleoylphosphatidylcholine.

1   4.   (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 each lipid vesicle further comprises a sterol.

1   5.   (Previously presented) The lipid vesicle formulation of claim 4, wherein

2 the sterol is cholesterol.

1   6.   (Previously presented) The lipid vesicle formulation of claim 4, wherein

2 the sterol is cholesterol and the amphipathic lipid is a phospholipid.

1   7.   (Previously presented) The lipid vesicle formulation of claim 6, wherein

2 the phospholipid is selected from the group consisting of phosphatidylcholine,

3 phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid,

4 palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine,

5 dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine,

6 and dilinoleoylphosphatidylcholine.

1   8.   (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 each lipid vesicle is a liposome.

1   9.   (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 each lipid vesicle is a lipid-nucleic acid particle.

1   10.   (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 each lipid vesicle is about 150 nm or less in diameter.

1   11.   (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 the cationic lipid only carries a positive charge at below physiological pH.

1   12.   (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 each lipid vesicle is about 100 nm or less in diameter.

1   13.   (Canceled)

1   14.   (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 at least 80% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

1   15.   (Previously presented) The lipid vesicle formulation of claim 1, wherein

2 about 90% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

PATENT

REMARKS

I.   STATUS OF THE CLAIMS

Upon entry of this amendment, claims 1-12, 14, and 15 are pending in this application and are presented for examination. Claim 13 has been canceled without prejudice to future prosecution. As such, no new matter has been introduced. Based on the following remarks, Applicants respectfully request reconsideration and allowance of the pending claims.

II.   REJECTION UNDER 35 U.S.C. § 103(a)

Claims 1-12, 14, and 15 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Saravolac *et al.* (US Patent No. 6,734,171) and Yoshioka *et al.* (US Patent No. 5,593,622). Applicants respectfully traverse.

In the Office Action, the Examiner states that she is not swayed by the Declaration of Dr. James Heyes under 37 C.F.R. § 1.132 submitted with the previous response because Applicants did not completely follow the method presented by Saravolac *et al.* and use ethanol in preparing the lipid stock solutions for the experiment described therein. *See,* Office Action at page 6. It is also unclear to the Examiner what the difference is between the method presented by Saravolac *et al.* and the method used in the experiment described in the Declaration that accounts for the difference in encapsulation efficiency. *See,* Office Action at page 8. Moreover, the Examiner alleges that Saravolac *et al.* provides motivation for one of skill in the art to make the instantly claimed lipid vesicles by teaching "lipid vesicles which comprise all of the same components which can encapsulate nucleic acids, which obviously includes mRNA because mRNA is made of nucleic acids, at efficiencies which are reported to be as high as 86% as is reported in Figure 8." *See,* Office Action at page 7.

In response, Applicants respectfully submit herewith a further Declaration of Dr. James Heyes under 37 C.F.R. § 1.132 (hereinafter, "Heyes Declaration") to present a ***head-to-head comparison*** between the encapsulation of plasmid DNA and mRNA following the method ***exactly*** as described in Example 1 of Saravolac *et al.* Dr. Heyes explains that the results of the ***direct comparison*** provide further evidence that the method described in Saravolac *et al.* for preparing lipid particles containing plasmid DNA is ***not suitable*** for producing the population of

lipid vesicles with an mRNA encapsulation efficiency as presently claimed. *See*, Heyes Declaration ¶ 7.

As explained by Dr. Heyes in his Declaration, he and his colleagues performed the ***head-to-head experiment*** using the optimal system shown in Figure 8 of Saravolac *et al.* with 34 mol% DODAC and 150 mM $NaPO_4$ in the absence of any added NaCl. *See*, Heyes Declaration ¶ 8. Notably, the experiment was performed using individual lipid stock solutions prepared in ***absolute ethanol***. *See*, Heyes Declaration ¶ 9.

According to Dr. Heyes, the results of the experiment (*see*, Table 1 of Heyes Declaration ¶ 10) show that ***less than half of the mRNA payload*** was successfully encapsulated into lipid particles prepared by the method described in Saravolac *et al*. *See*, Heyes Declaration ¶ 11. Dr. Heyes explains that the substantial amount of unencapsulated mRNA present in the formulation increases the likelihood of an unwanted immune response upon *in vivo* administration of the lipid particles. *See, id.* In contrast, Dr. Heyes points out that the amount of plasmid DNA successfully encapsulated into lipid particles was similar to the amount shown in Figure 8 of Saravolac *et al*. under the ***same formulation parameters***. *See, id.* Notably, the substantial difference in encapsulation efficiency observed for plasmid DNA and mRNA was ***not*** due to any difference in the method used, since a ***head-to-head comparison*** was performed following the method described in Saravolac *et al*. for ***both*** payloads. *See, id.*

Based on the results of the experiment, Dr. Heyes states that the method for preparing plasmid DNA lipid particles described in Saravolac *et al*. is ***not suitable*** for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed, wherein at least 70% of the mRNA in the formulation is fully encapsulated in the lipid vesicles. *See*, Heyes Declaration ¶ 12. Indeed, Dr. Heyes points out that ***only 43% of the mRNA payload*** was encapsulated following the method of Saravolac *et al*., despite using the ***exact*** reagents (*e.g.,* lipids dissolved in ***absolute ethanol***) and conditions for obtaining optimal formulations as described by Saravolac *et al*. *See, id.* Based on Tekmira Pharmaceuticals' clinical experience, and the scientific literature, Dr. Heyes notes that the mRNA lipid particles produced by the method of Saravolac *et al*. reported herein might invoke an unwanted innate immune response upon systemic administration to a human being. *See, id.* Thus, Dr. Heyes concludes that the

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Amdt. dated May 19, 2016
Reply to Final Office Action of April 15, 2016

PATENT

method described in Saravolac *et al.* is ***not amenable*** to the production of the population of lipid vesicles claimed in the present application. *See, id.*

For the foregoing reasons, Dr. Heyes submits that there is ***no motivation*** for one of ordinary skill in the art to take the teaching of Saravolac *et al.* and make a lipid vesicle formulation using mRNA and a specific combination of lipid components with any reasonable expectation that at least 70% of the mRNA in the formulation would be successfully encapsulated in the lipid vesicles. *See*, Heyes Declaration ¶13. In fact, Dr. Heyes points out that the skilled artisan would appreciate that Saravolac *et al.* only addresses the encapsulation of plasmid DNA, as all of the examples are directed to the preparation and use of plasmid DNA lipid particle formulations. *See, id.* As explained by Dr. Heyes, although it is true that mRNA is a type of nucleic acid, the skilled artisan would ***not*** consider mRNA to be equivalent to plasmid DNA in terms of its structure (*e.g.*, differences in number of strands and nucleotide composition) and properties (*e.g.*, difference in stability). *See, id.* As such, Dr. Heyes states that one of ordinary skill in the art would appreciate that the method described in Saravolac *et al.* is ***only*** applicable to plasmid DNA (and ***not*** to every type of nucleic acid), and the high encapsulation efficiency achieved for plasmid DNA using this method does ***not*** reasonably predict that mRNA will exhibit a similar encapsulation efficiency. *See, id.* Indeed, the ***head-to-head experiment*** described in the Heyes Declaration clearly shows that the method for preparing plasmid DNA lipid particles described in Saravolac *et al.* is ***not suitable*** for formulating the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed. *See, id.* Therefore, Dr. Heyes concludes that the results obtained for plasmid DNA using the method described in Saravolac *et al.* ***cannot*** be extrapolated to mRNA with a reasonable expectation that a comparable level of encapsulation would be achieved. *See, id.*

Applicants assert that the teaching of Yoshioka *et al.* does ***not*** remedy the deficiencies in the disclosure of Saravolac *et al.* In fact, Yoshioka *et al.* ***fails*** to provide any teaching whatsoever with regard to mRNA or the successful encapsulation and delivery thereof. Indeed, the Examiner merely relies on Yoshioka *et al.* for teaching the selection of cholesterol for inclusion in the lipid particles of Saravolac *et al. See,* Office Action at pages 5-6.

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   In view of the foregoing, Applicants assert that the cited references, whether alone or in combination, do **_not_** teach or suggest each of the features recited in the instant claims and thus fail to support a legal conclusion of obviousness. Indeed, **_none_** of these references discloses or suggests a lipid vesicle formulation of the present invention comprising a plurality of lipid vesicles and mRNA, wherein at least 70% of the mRNA in the formulation is fully encapsulated in the lipid vesicles. To the contrary, Applicants have provided a **_direct comparison_** between the encapsulation of plasmid DNA and mRNA following the method **_exactly_** as described in Saravolac _et al._ to demonstrate that this method for preparing plasmid DNA lipid particles is **_not_** **_suitable_** for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed. As explained by Dr. Heyes, one of skill in the art would appreciate that the method of Saravolac _et al._ is **_only_** applicable to plasmid DNA, and the high encapsulation efficiency achieved for plasmid DNA using this method does **_not_** reasonably predict that mRNA will exhibit a comparable level of encapsulation. The teaching of Yoshioka _et al._ does **_not_** remedy these deficiencies in the disclosure of Saravolac _et al._ Accordingly, Applicants respectfully request that the Examiner withdraw the present rejection under 35 U.S.C. § 103(a).

CONCLUSION

   In view of the foregoing, Applicants believe all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

   If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

            Respectfully submitted,

            /Joe C. Hao/

            Joe C. Hao
            Reg. No. 55,246

KILPATRICK TOWNSEND & STOCKTON LLP
Attachments

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 1042
Ian MacLachlan et al.	Examiner: Hirt, Erin E.
Application No.: 14/304,578	Art Unit: 1616
Filed: June 13, 2014	<u>DECLARATION UNDER 37 C.F.R. § 1.132</u>
For: LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY	
Customer No.: 20350	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, James Heyes, Ph.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1.   All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2.   I hold a Ph.D. (2001) in Medicinal Chemistry from the Institute of Cancer Research (Surrey, UK). I am presently the Senior Director of Formulation Chemistry at Arbutus Biopharma Corporation (Burnaby, Canada), formerly known as Tekmira Pharmaceuticals Corporation. The assignee of the above-referenced application, Protiva Biotherapeutics Inc., is a wholly-owned subsidiary of Arbutus Biopharma.

3.   My expertise lies in the development of lipid particle formulations and the design of novel compounds as components of lipid particles. A copy of my *Curriculum Vitae* is of record.

4.   I have reviewed the above-referenced patent application, and I am familiar with the contents therein. I have also reviewed the contents of the Office Action dated April 15, 2016.

5.   The present invention is directed to a lipid vesicle formulation comprising: (a) a plurality of lipid vesicles, wherein each lipid vesicle comprises: a cationic lipid; an amphipathic lipid; and a polyethyleneglycol (PEG)-lipid; and (b) messenger RNA (mRNA), wherein at least 70% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

6.   In the Office Action, the Examiner states that she is not swayed by my previous Declaration because we did not completely follow the method described in Saravolac *et al.* and use ethanol in preparing the lipid stock solutions for our experiment. *See,* Office Action at page 6. It is also unclear to the Examiner what the difference is between the method described in Saravolac *et al.* and the method used in our previous experiment that accounts for the difference in encapsulation efficiency. *See,* Office Action at page 8.

7.   I submit this Declaration to present a head-to-head comparison between the encapsulation of plasmid DNA and mRNA following the method exactly as described in Example 1 of Saravolac *et al.* The results of the direct comparison provide further evidence that the method described in Saravolac *et al.* for preparing lipid particles containing plasmid DNA is not suitable for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed.

8.   My colleagues and I performed the head-to-head experiment using the optimal system shown in Figure 8 of Saravolac *et al.* with 34 mol% DODAC and 150 mM NaPO$_4$ in the absence of any added NaCl.

9.   In particular, the experiment was performed as follows:

Preparation of Lipid Particles: DOPE, DODAC, and PEG$_{2000}$-CerC8 individual stock solutions were prepared at 10 mg/mL in absolute ethanol. Aliquots were combined to give

2

a molar ratio of DOPE:DODAC:PEG-CerC8 (51:34:15, mol%) to achieve a final lipid concentration of 5 mg/mL. Each formulation was prepared in duplicate, and tubes were placed under a stream of N_2 to evaporate off the solvent. Finally, they were exposed to vacuum to remove any trace amounts of the ethanol remaining. To the dried lipid film was added 100 µL of 1 M octyl glucopyranoside (OGP), 200 µL of 1.0 mg/mL luciferase mRNA or 1.0 mg/mL plasmid DNA, and 700 µL of the 150 mM $NaPO_4$ (no added NaCl). The tubes were then vigorously vortexed to solubilize the lipid films. Upon complete solubilization, the mixtures were allowed to stand for 30 minutes and then were transferred to dialysis tubing (3 mL Slide-A-Lyzers with MWCO of 10,000) and were dialyzed against 150 mM $NaPO_4$ (no added NaCl). The samples were dialyzed against 2 L of dialysis buffer over a 24 hour period with 2 changes of buffer over this time. Upon completion of dialysis, the samples were removed from the dialysis bags and analyzed for size and percent encapsulation.

10. The results of the experiment are summarized in Table 1. Three formulation parameters were measured, each in duplicate:

- % Encapsulation of mRNA or plasmid DNA (*i.e.*, the amount of mRNA or plasmid DNA that has been successfully encapsulated in the particle);
- Particle size; and
- Polydispersity (*i.e.*, a measure of the heterogeneity of sizes of particles in a mixture; a higher polydispersity index (PDI) indicates greater heterogeneity of particle sizes in the mixture).

Table 1. Size of particles and encapsulation of mRNA and plasmid DNA following dialysis

Payload	Replicate	Diameter (nm)	Polydispersity index (PDI)	% Encapsulation
Luciferase mRNA	1	116	0.45	43
	2	120	0.46	41
Plasmid DNA	1	197	0.52	91
	2	266	0.35	91

11.   Table 1 shows that less than half of the mRNA payload was successfully encapsulated into lipid particles prepared by the method described in Saravolac *et al.* The substantial amount of unencapsulated mRNA present in the formulation increases the likelihood of an unwanted immune response upon *in vivo* administration of the lipid particles. In contrast, the amount of plasmid DNA successfully encapsulated into lipid particles was similar to the amount shown in Figure 8 of Saravolac *et al.* under the same formulation parameters. Notably, the substantial difference in encapsulation efficiency observed for plasmid DNA and mRNA was not due to any difference in the method used, since a head-to-head comparison was performed following the method described in Saravolac *et al.* for both payloads.

12.   This experiment demonstrates that the method for preparing plasmid DNA lipid particles described in Saravolac *et al.* is not suitable for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed, wherein at least 70% of the mRNA in the formulation is fully encapsulated in the lipid vesicles. Indeed, only 43% of the mRNA payload was encapsulated following the method of Saravolac *et al.*, despite using the exact reagents (*e.g.,* lipids dissolved in absolute ethanol) and conditions for obtaining optimal formulations as described by Saravolac *et al.* Based on Tekmira Pharmaceuticals' clinical experience, and the scientific literature, the mRNA lipid particles produced by the method of Saravolac *et al.* reported herein might invoke an unwanted innate immune response upon systemic administration to a human being. Based on this experiment, I conclude that the method described in Saravolac *et al.* is not amenable to the production of the population of lipid vesicles claimed in the present application.

13.   For the foregoing reasons, I submit that there is no motivation for one of ordinary skill in the art to take the teaching of Saravolac *et al.* and make a lipid vesicle formulation using mRNA and a specific combination of lipid components with any reasonable expectation that at least 70% of the mRNA in the formulation would be successfully encapsulated in the lipid vesicles. In fact, the skilled artisan would appreciate that Saravolac *et al.* only addresses the encapsulation of plasmid DNA, as all of the examples are directed to the
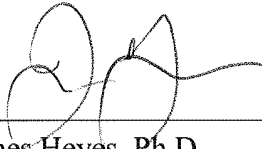
4

preparation and use of plasmid DNA lipid particle formulations. Although it is true that mRNA is a type of nucleic acid, the skilled artisan would not consider mRNA to be equivalent to plasmid DNA in terms of its structure (*e.g.,* differences in number of strands and nucleotide composition) and properties (*e.g.,* difference in stability). As such, one of ordinary skill in the art would appreciate that the method described in Saravolac *et al.* is only applicable to plasmid DNA (and not to every type of nucleic acid), and the high encapsulation efficiency achieved for plasmid DNA using this method does not reasonably predict that mRNA will exhibit a similar encapsulation efficiency. Indeed, our head-to-head experiment clearly shows that the method for preparing plasmid DNA lipid particles described in Saravolac *et al.* is not suitable for formulating the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed. Therefore, the results obtained for plasmid DNA using the method described in Saravolac *et al.* cannot be extrapolated to mRNA with a reasonable expectation that a comparable level of encapsulation would be achieved.

14.   The declarant has nothing further to say.

May 12, 2016
_____
Date

_____
James Heyes, Ph.D.

5

JOINT APPENDIX 26

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/304,578	06/13/2014	Ian MacLachlan	86399-001220US-911148	1042

20350   7590   10/03/2016
KILPATRICK TOWNSEND & STOCKTON LLP
Mailstop: IP Docketing - 22
1100 Peachtree Street
Suite 2800
Atlanta, GA 30309

EXAMINER
HIRT, ERIN E

ART UNIT	PAPER NUMBER
1616	

NOTIFICATION DATE	DELIVERY MODE
10/03/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipefiling@kilpatricktownsend.com
jlhice@kilpatrick.foundationip.com

PTOL-90A (Rev. 04/07)

Office Action Summary	Application No.	Applicant(s)
	14/304,578	MACLACHLAN ET AL.
	Examiner	Art Unit	AIA (First Inventor to File) Status
	ERIN HIRT	1616	No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE _3_ MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.
- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1) ☒ Responsive to communication(s) filed on _05/19/16_.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.
2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☒ Claim(s) _1-12,14 and 15_ is/are pending in the application.
   5a) Of the above claim(s) _____ is/are withdrawn from consideration.
6) ☐ Claim(s) _____ is/are allowed.
7) ☒ Claim(s) _1-12,14 and 15_ is/are rejected.
8) ☐ Claim(s) _____ is/are objected to.
9) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined <u>allowable</u>, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10) ☐ The specification is objected to by the Examiner.
11) ☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.
   Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
   Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   Certified copies:
      a)☐ All   b)☐ Some** c)☐ None of the:
      1.☐ Certified copies of the priority documents have been received.
      2.☐ Certified copies of the priority documents have been received in Application No. _____.
      3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.
3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
4) ☐ Other: _____.

U.S. Patent and Trademark Office
PTOL-326 (Rev. 11-13)   Office Action Summary   Part of Paper No./Mail Date 20160927

JA00710

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The present application is being examined under the pre-AIA first to invent

provisions.

DETAILED ACTION

Continued Examination Under 37 CFR 1.114

A request for continued examination under 37 CFR 1.114, including the fee set

forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this

application is eligible for continued examination under 37 CFR 1.114, and the fee set

forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action

has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on

05/19/16 has been entered.

Status of Action

Currently claims 1-12 and 14-15 are pending in this application. Claim 13 was

cancelled.

Status of Claims

Accordingly, new claims 1-12 and 14-15 are presented for examination on the

merits for patentability. Rejection(s) not reiterated from the previous Office Action are

hereby withdrawn. The following rejections are either reiterated or newly applied. They

constitute the complete set of rejections presently being applied to the instant

application.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created

doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the

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unjustified or improper timewise extension of the "right to exclude" granted by a patent

and to prevent possible harassment by multiple assignees. A nonstatutory double

patenting rejection is appropriate where the conflicting claims are not identical, but at

least one examined application claim is not patentably distinct from the reference

claim(s) because the examined application claim is either anticipated by, or would have

been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46

USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed.

Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*,

686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619

(CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d)

may be used to overcome an actual or provisional rejection based on nonstatutory

double patenting provided the reference application or patent either is shown to be

commonly owned with the examined application, or claims an invention made as a

result of activities undertaken within the scope of a joint research agreement. See

MPEP § 717.02 for applications subject to examination under the first inventor to file

provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) -

706.02(l)(3) for applications not subject to examination under the first inventor to file

provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR

1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be

used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application

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in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26,

PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may

be filled out completely online using web-screens. An eTerminal Disclaimer that meets

all requirements is auto-processed and approved immediately upon submission. For

more information about eTerminal Disclaimers, refer to

www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

     Claims 1-12 and 14-15 are rejected on the ground of nonstatutory double

patenting as being unpatentable over claims 1 and 4-13 of U.S. Patent No. 8329070

('070). Although the claims at issue are not identical, they are not patentably distinct

from each other because the instant application and '070 claim related inventions.

Specifically, the instant application is claiming the lipid vesicles that are formed by the

apparatus of '070 because '070 teaches wherein the lipid vesicles encapsulate a nucleic

acid and mRNA is formed of nucleic acids and both '070 and the instant application

teach wherein the lipid vesicles comprise PEG-lipids, cholesterol, phospholipids and a

cationic lipid. As such, it would have been obvious to one of ordinary skill in the art that

the apparatus for making the claimed lipid vesicles would render said lipid vesicles

obvious.

     Claims 1-12 and 14-15 are rejected on the ground of nonstatutory double

patenting as being unpatentable over claims 1-7 and 10-22 of U.S. Patent No. 7901708

('708). Although the claims at issue are not identical, they are not patentably distinct

from each other because the instant application and '708 claim related inventions.

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Specifically, the instant application is claiming the lipid vesicles that are formed by the

process of '708 because '708 teaches wherein the produced lipid vesicles encapsulate

a nucleic acid and mRNA is formed of nucleic acids and both '708 and the instant

application teach wherein the lipid vesicles comprise PEG-lipids, cholesterol,

phospholipids and a cationic lipid, and wherein the encapsulation efficiency is the same

as that which is instantly claimed, e.g. greater than 70%. As such, it would have been

obvious to one of ordinary skill in the art that the process for making the claimed lipid

vesicles would render said lipid vesicles obvious, because if the process for making the

lipid vesicles is known then the lipid vesicles themselves were already known in the art.

Claims 1-12 and 14-15 are rejected on the ground of nonstatutory double

patenting as being unpatentable over claims 57-58, 60, 63-70, 72, 75-82 of recently

allowed application 13/684066 ('066). Although the claims at issue are not identical,

they are not patentably distinct from each other because the instant application and '066

claim related inventions. Specifically, the instant application is claiming the lipid vesicles

that are formed by the process of '066 because '066 teaches wherein the produced lipid

vesicles encapsulate a nucleic acid, specifically mRNA and both '066 and the instant

application teach wherein the lipid vesicles comprise PEG-lipids, cholesterol,

phospholipids and a cationic lipid, and '066 further discloses wherein the encapsulation

efficiency is the same as that which is instantly claimed, e.g. greater than 70%. As such,

it would have been obvious to one of ordinary skill in the art that the process for making

the claimed lipid vesicles would render said lipid vesicles obvious, because if the

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process for making the lipid vesicles is known then the lipid vesicles themselves were

already known in the art.

Response to Arguments/Remarks

Applicant's arguments, filed 05/19/16, especially applicant's affidavit also

submitted 05/19/16 have overcome the previous 103 rejections which are hereby

withdrawn by the examiner. Specifically, applicants demonstrated that the primary prior

Saravolac does not encapsulate mRNA with the same efficiency as other nucleic acids

and as such does not function as effectively the claimed lipid vesicles which applicants

have demonstrated do effectively encapsulate mRNA with efficiencies of greater than

70%. However, in reviewing the literature the examiner discovered the original examiner

did not restrict the lipid vesicles from the apparatus and method claims and as such

there are double patenting rejections which were inadvertently previously missed by the

examiner.

Declaration under 1.132

James Heyes provided a declaration under 1.132, filed May 19, 2016 and dated

05/12/16. The Declaration meets the formal requirements. In the most relevant part, the

Declaration demonstrates that the prior art, Saravolac does not encapsulate mRNA with

the efficiency that is instantly claimed in their lipid vesicles. Specifically, Saravolac

reports encapsulating nucleic acids with efficiencies of greater than 70%. However,

when Dr. Heyes performed the method of Saravolac this high encapsulation efficiency

was only observed with plasmid DNA (reported encapsulation efficiencies of 91%),

whereas when mRNA was encapsulated the efficiency was only about 42%. A

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Declaration is due full consideration and weight for all that it discloses. Declarations are

reviewed for the following considerations: 1) whether the Declaration presents a nexus

such as a side-by-side or single-variable comparison (In re Huang, 40 USPQ2d 1685,

1689 (Fed. Cir. 1996)), 2) whether the Declaration presents a comparison to the closest

art, 3) whether the Declaration is commensurate in scope with the scope of the claims

(In re Kulling, 14 USPQ2d 1056, 1058 (Fed. Cir. 1990)), 4) whether the Declaration

shows a difference in kind rather than merely a difference in degree (In re Waymouth,

182 USPQ 290, 293 (C.C.P.A. 1974)), and 5) whether the prima facie case is

sufficiently strong that allegedly superior results are insufficient to overcome the case

for obviousness (Pfizer Inc. v. Apotex, Inc., 82 USPQ2d 1321, 1339 (Fed. Cir. 2007)).

The relevant criterion here is No. 5, whether the prima facie case is sufficiently strong

that allegedly superior results are insufficient to overcome the case for obviousness.

The examiner has carefully reviewed the Declaration, including the data presented in

the Declaration. The data shows that the prior art method does not produce lipid

vesicles which encapsulated greater than 70% of the mRNA as applicant's have

demonstrated they are able to do with their lipid vesicles. As such, the 103 rejection

over Saravolac is overcome and is hereby withdrawn by the examiner.

Conclusion

Claims 1-12 and 14-15 are rejected.

Any inquiry concerning this communication or earlier communications from the

examiner should be directed to ERIN HIRT whose telephone number is (571)270-1077.

The examiner can normally be reached on Monday through Friday 9:30am to 6:30pm.

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If attempts to reach the examiner by telephone are unsuccessful, the examiner's

supervisor, Sue X. Liu can be reached on 571-272-5539. The fax phone number for the

organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the

Patent Application Information Retrieval (PAIR) system. Status information for

published applications may be obtained from either Private PAIR or Public PAIR.

Status information for unpublished applications is available through Private PAIR only.

For more information about the PAIR system, see http://pair-direct.uspto.gov. Should

you have questions on access to the Private PAIR system, contact the Electronic

Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a

USPTO Customer Service Representative or access to the automated information

system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Erin Hirt/
Examiner, Art Unit 1616

JOINT APPENDIX 27

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

20350 7590 10/19/2016		

KILPATRICK TOWNSEND & STOCKTON LLP
Mailstop: IP Docketing - 22
1100 Peachtree Street
Suite 2800
Atlanta, GA 30309

EXAMINER
HIRT, ERIN E

ART UNIT	PAPER NUMBER
1616	

DATE MAILED: 10/19/2016

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/304,578	06/13/2014	Ian MacLachlan	86399-001220US-911148	1042

TITLE OF INVENTION: LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	01/19/2017

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED.   THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT.   SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED.   THIS STATUTORY PERIOD CANNOT BE EXTENDED.   SEE 35 U.S.C. 151.   THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION.   IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PTOL-85 (Rev. 02/11)

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: <u>Mail</u>   Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or <u>Fax</u>   (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

20350   7590   10/19/2016

KILPATRICK TOWNSEND & STOCKTON LLP
Mailstop: IP Docketing - 22
1100 Peachtree Street
Suite 2800
Atlanta, GA 30309

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

_____ (Depositor's name)
_____ (Signature)
_____ (Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/304,578	06/13/2014	Ian MacLachlan	86399-001220US-911148	1042

TITLE OF INVENTION: LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	01/19/2017

EXAMINER	ART UNIT	CLASS-SUBCLASS
HIRT, ERIN E	1616	424-450000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE   (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) :   ☐ Individual   ☐ Corporation or other private group entity   ☐ Government

4a. The following fee(s) are submitted:
☐ Issue Fee
☐ Publication Fee (No small entity discount permitted)
☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
☐ A check is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☐ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
☐ Applicant certifying micro entity status. See 37 CFR 1.29

☐ Applicant asserting small entity status. See 37 CFR 1.27

☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____   Date _____

Typed or printed name _____   Registration No. _____

Page 2 of 3

JA00719
GENV-00011633

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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		EXAMINER
20350	7590	10/19/2016

KILPATRICK TOWNSEND & STOCKTON LLP
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Suite 2800
Atlanta, GA 30309

EXAMINER
HIRT, ERIN E

ART UNIT	PAPER NUMBER
1616	

DATE MAILED: 10/19/2016

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

PTOL-85 (Rev. 02/11)

JA00720
GENV-00011634

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:
1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

	Application No.	Applicant(s)
Notice of Allowability	14/304,578	MACLACHLAN ET AL.
	Examiner	Art Unit	AIA (First Inventor to File) Status
	ERIN HIRT	1616	No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *10/4/16*.

   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on_____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are *1-12,14 and 15*. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All   b) ☐ Some   *c) ☐ None of the:

       1. ☐ Certified copies of the priority documents have been received.

       2. ☐ Certified copies of the priority documents have been received in Application No. _____ .

       3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application. **THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.**

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.

   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)

2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____

3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material

4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .

5. ☐ Examiner's Amendment/Comment

6. ☒ Examiner's Statement of Reasons for Allowance

7. ☐ Other _____.

/Mina Haghighatian/
Primary Examiner, Art Unit 1616

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Art Unit: 1616

The present application is being examined under the pre-AIA first to invent

provisions.

REASONS FOR ALLOWANCE

The following is an examiner's statement of reasons for allowance: Applicants

have filed e-terminal disclaimers over U.S. Patents 7901708, 8329070, and Application

No. 13684066 which have been accepted. As such all previous double patenting

rejections are hereby withdrawn by the examiner. The examiner has searched this case

several times and applicants have been able to demonstrate that their method of

making liposomes allows for liposomes which more effectively encapsulate mRNA over

the methods/liposomes of the prior art and have shown this effective encapsulation

through the submission of several declarations under 1.132. No further art was found

which had these high encapsulation efficiencies of mRNA. Thus, the instant claims are

now allowable.

Any comments considered necessary by applicant must be submitted no later

than the payment of the issue fee and, to avoid processing delays, should preferably

accompany the issue fee. Such submissions should be clearly labeled "Comments on

Statement of Reasons for Allowance."

Conclusion

Claims 1-12, 14 and 15 are allowed.

Any inquiry concerning this communication or earlier communications from the

examiner should be directed to ERIN HIRT whose telephone number is (571)270-1077.

The examiner can normally be reached on Monday through Friday 9:30am to 6:30pm.

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If attempts to reach the examiner by telephone are unsuccessful, the examiner's

supervisor, Sue X. Liu can be reached on 571-272-5539. The fax phone number for the

organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the

Patent Application Information Retrieval (PAIR) system. Status information for

published applications may be obtained from either Private PAIR or Public PAIR.

Status information for unpublished applications is available through Private PAIR only.

For more information about the PAIR system, see http://pair-direct.uspto.gov. Should

you have questions on access to the Private PAIR system, contact the Electronic

Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a

USPTO Customer Service Representative or access to the automated information

system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Erin Hirt
Examiner, Art Unit 1616

/Mina Haghighatian/
Primary Examiner, Art Unit 1616

JOINT APPENDIX 28

UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE PATENT TRIAL AND APPEAL BOARD

_____

MODERNA THERAPEUTICS, INC.,
Petitioner,

v.

PROTIVA BIOTHERAPEUTICS, INC.,
Patent Owner.

_____

Case IPR2018-00739
Patent 9,364,435 B2

_____

Before SHERIDAN K. SNEDDEN, SUSAN L. C. MITCHELL, and
RICHARD J. SMITH, *Administrative Patent Judges*.

MITCHELL, *Administrative Patent Judge*.

FINAL WRITTEN DECISION

Determining Claims 1–6, 9, 12, 14, and 15
Unpatentable in *Inter Partes* Review
35 U.S.C. § 318(a) and 37 C.F.R. § 42.73

Determining Claims 7, 8, 10, 11, 13, and 16–20
Not Unpatentable in *Inter Partes Review*
35 U.S.C. § 318(a) and 37 C.F.R. § 42.73

Denying Patent Owner's Motion to Amend
35 U.S.C. § 316(d) and 37 C.F.R. § 42.121

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Patent 9,364,435 B2

I.   INTRODUCTION

This is a final written decision in *inter partes* review of claims 1–20 of U.S. Patent No. 9,364,435 B2 (Ex. 1001, "the '435 patent") entered pursuant to 35 U.S.C. § 318(a) and 37 C.F.R. § 42.73. For the reasons set forth below, we determine that Petitioner has shown by a preponderance of the evidence that claims 1–6, 9, 12, 14, and 15 of the '435 patent are unpatentable under 35 U.S.C. § 102. *See* 35 U.S.C. § 316(e). We also determine that Petitioner has not shown by a preponderance of the evidence that claims 7, 8, 10, 11, 13, or 16–20 are unpatentable.

Because we have found only some of the challenged claims unpatentable, we address Patent Owner's contingent Motion to Amend concerning proposed substitute claims for those unpatentable claims, which are proposed substitute claims 21–26, 29, 32, 34, and 35. We also find that Patent Owner's proposed substitute claims 21–26, 29, 32, 34, and 35 are unpatentable. Therefore, we deny Patent Owner's Motion to Amend.

A.   *Procedural History*

Moderna Therapeutics, Inc. ("Petitioner")[1] filed a Petition to institute an *inter partes* review of claims 1–20 (the "challenged claims") of the '435 patent. Paper 2 ("Pet."); *see* 35 U.S.C. §§ 311–319. Petitioner relied upon the Declaration of Andrew S. Janoff, Ph.D. to support its challenge.

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[1] Petitioner states that the name of its parent has been changed to Moderna, Inc., and that Moderna, Inc.'s intellectual property matters are now conducted under the name of ModernaTX, Inc., which is a fully-owned subsidiary of Moderna, Inc. Paper 46, 2.

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See generally Pet. Protiva Biotherapeutics, Inc. ("Patent Owner")[2] filed a
Preliminary Response to the Petition. Paper 12 ("Prelim. Resp.").

> Pursuant to 35 U.S.C. § 314(a), on September 12, 2018, we instituted
an *inter partes* review of challenged claims 1–20 (Paper 15, "Inst. Dec." or
"Institution Decision") instituting *inter partes* review of all challenged
claims under all asserted grounds. Inst. Dec. 33. Patent Owner filed a
Response (Paper 24, "PO Resp.") supported by the Declaration of David H.
Thompson, Ph.D (Ex. 2009). Petitioner filed a Reply (Paper 28, "Reply")
supported by a second Declaration of Dr. Janoff (Ex. 1021), and Patent
Owner filed an authorized Sur-reply (Paper 34, "Sur-reply"). *See* Papers 16,
19 (authorizing Patent Owner's Sur-Reply).

> Patent Owner filed a contingent motion to amend (Paper 26
(corrected), "Mot.") supported by a Declaration of Dr. Thompson
(Ex. 2040), which Petitioner opposed (Paper 29, "Opposition to Motion to
Amend") with a supporting Declaration of Dr. Janoff (Ex. 1020). Patent
Owner filed a Reply to Petitioner's opposition. Paper 33, "Reply Opp."

> At the request of both parties, we held an oral hearing on June 6,
2019, and the transcript of that hearing has been entered into the record.
Paper 49 ("Tr.").

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[2] According to Patent Owner, Protiva Biotherapeutics, Inc. ("Protiva")
existed as a wholly-owned subsidiary of Arbutus Biopharma Corporation
and was amalgamated into Arbutus Biopharma Corporation in January 2018.
Paper 14, 2. Patent Owner identifies Arbutus Biopharma Corporation (fka
"Tekmira"), Genevant Sciences, Ltd., and its fully owned subsidiaries:
Genevant Sciences Holding, Ltd., Genevant Sciences Corporation, Genevant
Sciences, Inc., and Genevant Sciences, GmbH, as the real parties in interest.
Id.

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B.   Related Proceedings

Patent Owner identifies the following related matters:

Moderna Therapeutics, Inc. v. Protiva Biotherapeutics, Inc.,
IPR2018-00680 regarding U.S. Patent No. 9,404,127 B2; and European
Patent Office Opposition proceedings regarding EP 2 279 254. Paper 14, 2.

C.   The '435 Patent (Ex. 1001)

The '435 patent relates to "stable nucleic acid-lipid particles (SNALP)
comprising a nucleic acid (such as one or more interfering RNA), methods
of making the SNALP, and methods of delivering and/or administering the
SNALP." Ex. 1001, Abstract. The '435 patent states that "[t]he present
invention is based, in part, upon the surprising discovery that lipid particles
comprising from about 50 mol % to about 85 mol % of a cationic lipid, from
about 13 mol% to about 49.5 mol % of a non-cationic lipid, and from about
0.5 mol % to about 2 mol % of a lipid conjugate provide advantages when
used for the in vitro or in vivo delivery of an active agent, such as a
therapeutic nucleic acid (e.g., an interfering RNA)." *Id.* at 5:55–62. The
'435 patent further states that

> the present invention provides stable nucleic acid-lipid particles
> (SNALP) that advantageously impart increased activity of the
> encapsulated nucleic acid (e.g., an interfering RNA such as
> siRNA) and improved tolerability of the formulations in vivo,
> resulting in a significant increase in the therapeutic index as
> compared to nucleic acid-lipid particle compositions previously
> described. Additionally, the SNALP of the invention are stable
> in circulation, e.g., resistant to degradation by nucleases in serum
> and are substantially non-toxic to mammals such as humans.

Id. at 5:62–6:5.

The '435 patent identifies specific SNALP formulations that
encapsulate siRNA as the nucleic acid, such as the 1:57 SNALP and the 1:62

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SNALP, and states that "the Examples herein illustrate that the improved lipid particle formulations of the invention are highly effective in downregulating the mRNA and/or protein levels of target genes." *Id*. at 6:5–30.

D.   *Illustrative Claim*

Petitioner challenges claims 1–20 of the '435 patent. Claim 1 is illustrative and reproduced below:

> 1. A nucleic acid-lipid particle comprising:
> (a) a nucleic acid;
> (b) a cationic lipid comprising from 50 mol % to 85 mol % of the total lipid present in the particle;
> (c) a non-cationic lipid comprising from 13 mol % to 49.5 mol % of the total lipid present in the particle; and
> (d) a conjugated lipid that inhibits aggregation of particles comprising from 0.5 mol % to 2 mol % of the total lipid present in the particle.

Ex. 1001, 89:55–63.

Claim 1 is the only independent claim, and claims 2–20 are directly or indirectly dependent on claim 1. *Id*. at 89:55–92:22.

E.   *The Instituted Grounds of Unpatentability*

We instituted the instant trial based on the following grounds of unpatentability. Inst. Dec. 5, 33.

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Reference[s]	Basis	Claims challenged
WO 2005/007196 A2[3] and US 2006/0134189 A1[4]	§ 103	1–20
'196 PCT, '189 Publication, Lin,[5] and Ahmad[6]	§ 103	1–20
US 2006/0240554 A1[7]	§§ 102 and 103	1–20

II.   ANALYSIS

A.   *Person of Ordinary Skill in the Art*

Petitioner asserts that a person having ordinary skill in the art ("POSITA") "would have specific experience with lipid particle formation and use in the context of delivering therapeutic payloads, and would have a Ph.D., an M.D., or a similar advanced degree in an allied field (*e.g.*, biophysics, microbiology, biochemistry) or an equivalent combination of education and experience." Pet. 5 (citing Ex. 1007 ¶¶ 31–32). Petitioner further states that "[t]his level of skill is representative of the inventors on the '435 patent and authors/inventors of prior art cited herein." *Id.* at 6. We

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[3] Ian MacLachlan et al., WO 2005/007196 A2, published Jan. 27, 2005 ("'196 PCT"). Ex. 1002.

[4] Ian MacLachlan et al., US 2006/0134189 A1, published June 22, 2006 ("'189 Publication"). Ex. 1003.

[5] Alison J. Lin et al., *Three-Dimensional Imaging of Lipid Gene-Carriers: Membrane Charge Density Controls Universal Transfection Behavior in Lamellar Cationic Liposome-DNA Complexes*, 84 BIOPHYSICAL J. 3307–16 (2003) ("Lin"). Ex. 1005.

[6] Ayesha Ahmad et al., *New Multivalent Cationic Lipids Reveal Bell Curve for Transfection Efficiency Versus Membrane Charge Density: Lipid-DNA Compleses for Gene Delivery*, 7 J. GENE MED. 739–48 (2005) ("Ahmad"). Ex. 1006.

[7] Tongqian Chen et al., US 2006/0240554 A1, published Oct. 26, 2006 ("'554 Publication"). Ex. 1004.

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applied that description for purposes of our Institution Decision. Inst.
Dec. 7.

Patent Owner objects to Petitioner's proffered definition for two
reasons. PO Resp. 9–10 (citing Ex. 2009 ¶¶ 22–24). First, Patent Owner
objects to Petitioner's equating the level of ordinary skill with the level of
skill of the inventors of the '435 patent. *Id.* According to Patent Owner,
"the petition has improperly assumed a much higher level of skill than that
of a person of ordinary skill in the art ("POSITA")." *Id.* at 10 (citing
Ex. 1007 ¶ 31; 2009 ¶¶ 22–24; Ex. 2028, 44:8–12). Patent Owner further
states that "[b]ecause the petition sets the level much higher, to that of the
inventors, Petitioner has failed to conduct an appropriate analysis." *Id*. at 10
(citing Ex. 2009 ¶¶ 23–24).

As an initial matter, we did not rely on Petitioner's statement that the
proposed level of skill is representative of the inventors on the '435 patent in
our Institution Decision, and we do not rely on it for purposes of this
Decision. *See* Inst. Dec. 6. We do not view Petitioner's statement regarding
the proposed level of ordinary skill as representative of the inventors on the
'435 patent as part of Petitioner's proposed level of ordinary skill in the art.

Second, Patent Owner objects to Petitioner's definition of a person of
ordinary skill because it is indeterminable. PO Resp. 10. Patent Owner
bases this contention on its characterization of Dr. Janoff's testimony during
cross-examination. *Id.* Patent Owner states that Dr. Janoff "repeatedly
indicat[ed] that Petitioner's own definition is 'too vague' to understand." *Id.*
(citing Ex. 2028, 33:6–14, 34:7–35:25, 36:1–37:5). Although Dr. Janoff
may not have been as responsive to some questions during his deposition as
may have been appropriate, *see* PO Resp. 4–8, what Dr. Janoff said in the
cited portions of his testimony was that the *questions* from Patent Owner's

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counsel were "too vague." *See* Ex. 2028, 3:36–14, 34:7–35:25, 36:1–37:5.

The cited portions of Dr. Janoff's testimony indicate that, rather than

specifically asking about Petitioner's proposed definition of a person having

ordinary skill in the art, Dr. Janoff was asked questions about his work

experience. *Id.*; *see, e.g., id.* at 33:7–8, 35:7–8 ("Do you have specific

experience working with lipid particles?" "Do you have any experience with

any therapeutic payload?").

Although Patent Owner cites to Dr. Thompson's testimony in support

of its objections to Petitioner's definition, it does not expressly proffer its

own definition of a person of ordinary skill in the art. PO Resp. 9–10 (citing

Ex. 2009 ¶¶ 22–24). Dr. Thompson states that "Dr. Janoff has not simply

applied a slightly higher level of skill in the art in setting forth his opinions

in his declaration, but has assumed a much higher level of skill than that of a

person of ordinary skill in the art." Ex. 2009 ¶ 24. Dr. Thompson, however,

does not proffer what he considers to be an appropriate level of ordinary

skill in the art. *See generally* Ex. 2009.

Accordingly, we find on the record as a whole that a person of

ordinary skill in the art would have specific experience with, and/or be

generally familiar with, lipid particle formation and use in the context of

delivering therapeutic payloads, and would have a Ph.D., an M.D., or a

similar advanced degree in an allied field (*e.g.*, biophysics, microbiology,

biochemistry) or an equivalent combination of education and experience.

We also find on this record that both Dr. Janoff and Dr. Thompson are ones

of at least ordinary skill in the art under this standard. *See* Ex. 1007 ¶¶ 8–22;

Ex. 1018 (*curriculum vitae* of Dr. Janoff); Ex. 2009 ¶¶ 1–6; Ex. 2010

(*curriculum vitae* of Dr. Thompson).

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We further note that the prior art itself demonstrates the level of skill in the art at the time of the invention. *See Okajima v. Bourdeau*, 261 F.3d 1350, 1355 (Fed. Cir. 2001) (explaining that specific findings regarding ordinary skill level are not required "where the prior art itself reflects an appropriate level and a need for testimony is not shown") (quoting *Litton Indus. Prods., Inc. v. Solid State Sys. Corp.*, 755 F.2d 158, 163–64 (Fed. Cir. 1985)).

B.   Claim Construction

For petitions filed before November 13, 2018, [8], the Board interprets claim terms in an unexpired patent according to the broadest reasonable construction in light of the specification of the patent in which they appear. 37 C.F.R. § 42.100(b); *Cuozzo Speed Techs., LLC v. Lee*, 136 S. Ct. 2131, 2142 (2016) (affirming applicability of broadest reasonable construction standard to *inter partes* review proceedings). "Under a broadest reasonable interpretation, words of the claim must be given their plain meaning, unless such meaning is inconsistent with the specification and prosecution history." *Trivascular, Inc. v. Samuels*, 812 F.3d 1056, 1062 (Fed. Cir. 2016). Any special definitions for claim terms must be set forth with reasonable clarity, deliberateness, and precision. *See In re Paulsen*, 30 F.3d 1475, 1480 (Fed. Cir. 1994). Only terms in controversy must be construed and only to the extent necessary to resolve the controversy.

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[8] The Petition was filed March 5, 2018. Paper 2. *See* Changes to the Claim Construction Standard for Interpreting Claims in Trial Proceedings Before the Patent Trial and Appeal Board, 83 Fed. Reg. 51,340 (Oct. 11, 2018) (to be codified at 37 C.F.R. pt. 42).

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Vivid Techs., Inc. v. Am. Sci. & Eng'g, Inc., 200 F.3d 795, 803 (Fed. Cir. 1999).

As in our Decision on Institution, because Patent Owner substantively challenges the proposed construction of the term "nucleic acid-lipid particle" and relies on its narrower definition of the term in its arguments addressing the grounds asserted, we address the construction of that term, but find that we need not construe any other terms addressed by Petitioner for the purpose of reaching our institution decision. We again note, however, that Petitioner provides the same definition for "cationic lipid" as the express definition set forth in the specification of the '435 patent, namely, "any of a number of lipid species that carry a net positive charge at a selected pH, such as physiological pH (e.g., pH of about 7.0)." *Compare* Pet. 24, *with* Ex. 1001, 12:59–61.

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nucleic acid-lipid particle

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In our Decision on Institution, we expressly defined the term "nucleic acid-lipid particle. *See* Inst. Dec. 7–11. In construing this claim term when read in light of the Specification of the '435 patent, we stated that it should not be limited to the definition of a stable nucleic acid-lipid particle or SNALP and that it should not be limited to *in vivo* use. *See* Inst. Dec. 9–10. We concluded that:

> Our preliminary construction of "nucleic acid-lipid particle" at this stage of the proceeding and for purposes of this decision is derived from the express definition of "lipid particle" as set forth in the '435 patent that generally describes use of such a lipid particle to deliver nucleic acid as an active or therapeutic agent where the nucleic acid may be encapsulated in the lipid to protect it from enzymatic degradation. At this stage of the proceeding, we define "nucleic acid-lipid particle" as "a particle that comprises a nucleic acid and lipid, in which the nucleic acid

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> may be encapsulated in the lipid portion of the particle." *See* Ex. 1001, 11:14–22.

Inst. Dec. 10–11.

Patent Owner asserts that our proposed construction is too broad "at least to the extent [that it encompasses] lipid particles lacking any encapsulated nucleic acid." PO Resp. 11. Patent Owner asserts that a "nucleic acid-lipid particle" when read in light of the Specification of the '435 patent requires that the nucleic acid be encapsulated in the lipid particle. *Id*. at 11–12. Patent Owner's reasoning is as follows.

> A "nucleic acid-lipid particle" expressly includes a nucleic acid. According to the '435 patent, "nucleic acids, when present in the lipid particles of the present invention, are resistant in aqueous solution to degradation with a nuclease." EX1001, 11:51–54. The '435 patent describes nucleic acid encapsulation in the lipid particle as conferring resistance to such enzymatic degradation. EX1001, 11:20–22; *see also* EX2007, 4:15–19; 22:40–45; 23:1–3; 23:27–29; 26:35–37. A "lipid particle" "*may* [include a nucleic acid] encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation." EX1001, 11:14–22. A "nucleic acid-lipid particle," however, *does* include a nucleic acid encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation. EX1001, 11:23–31, 11:51–54; *see also* EX2009 ¶39.

PO Resp. 11–12 (emphasis in original).

In short, Patent Owner asserts that we should construe "nucleic acid-lipid particle" as a SNALP. *Id*. at 12–13 (citing testimony of Dr. Janoff and Dr. Thompson and the Specification of the '435 patent). Patent Owner concludes, however, that whichever construction we choose as the broadest reasonable construction, "the petition fails to establish the unpatentability of claims 1–20." PO Resp. 13.

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Petitioner responds that Patent Owner is trying inappropriately to import "serum stable" and "systemic use" limitations into the claims to limit the claims to a SNALP. *See* Reply 3–5.

We find that our construction of "nucleic acid-lipid particle" is the broadest reasonable construction in light of the Specification of the '435 patent. 37 C.F.R. § 42.100(b); *Cuozzo Speed Techs.,* 136 S. Ct. at 2142. For instance, the '435 patent identifies a "stable nucleic acid-lipid particle" or SNALP as an example of a "nucleic acid-lipid particle," *see, e.g.*, Ex. 1001, 3:38–39 (stating "nucleic acid-lipid particle (e.g., SNALP)"), 3:47–48, 3:57–58, 4:4–8, 4:12–13, 4:17–19, 27:43–45, and the term "nucleic acid-lipid particle" is broader than a SNALP.

The Specification of the '435 patent states that a SNALP *requires* the nucleic acid to be fully encapsulated within the lipid. *See* Ex. 1001, 11:23–30. A "lipid particle" with a nucleic acid as a therapeutic agent, i.e., a nucleic acid-lipid particle, however, *may* have the nucleic acid "encapsulated in the lipid, thereby protecting the agent [or nucleic acid] from enzymatic degradation." *Id*. at 11:14–22 (defining "lipid particle"). Encapsulation, or more specifically, full encapsulation of the nucleic acid is not required according to the Specification of the '435 patent.

In fact, "lipid encapsulated" as defined in the Specification of the '435 patent "can refer to a lipid particle that provides an active agent or therapeutic agent, such as a nucleic acid (e.g., an interfering RNA), with full encapsulation, partial encapsulation, or both. In a preferred embodiment, the nucleic acid is fully encapsulated in the lipid particle (e.g., to form an SPLP, pSPLP, SNALP, or other nucleic acid-lipid particle)." *Id*. at 11:59–64. The Specification of the '435 patent expressly states that only "[i]n some

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embodiments, the nucleic acid is fully encapsulated in the lipid particle." *Id.* at 27:43–47.

Dr. Thompson attempts to shoehorn the statement that nucleic acids, when in the lipid particles, "are resistant in aqueous solution to degradation with a nuclease," to require a "nucleic acid-lipid particle" as required by the claims to be a SNALP. *See* PO Resp. 11–12; Ex. 2009 ¶¶ 39–45 (stating in paragraph 45 that "there is no meaningful distinction between a nucleic acid-lipid particle and a SNALP in the context of the '435 patent"). Although we do not question that the '435 patent touts SNALPs as the focus of the '435 patent, *see* Ex. 1001, Abst., the claims are not limited to SNALPs when the claims are read in light of the Specification of the '435 patent.

Accordingly, we construe "nucleic acid-lipid particle" as "a particle that comprises a nucleic acid and lipids, in which the nucleic acid may be encapsulated in the lipid portion of the particle." *See* Ex. 1001, 15:52–63.[9] This is the same construction that we applied for purposes of the Institution Decision. Inst. Dec. 10–11.

We determine that we need not expressly construe any undisputed terms. *See Nidec Motor Corp. v. Zhongshan Broad Ocean Motor Co.*, 868 F.3d 1013, 1017 (Fed. Cir. 2017) ("[W]e need only construe terms 'that are in controversy, and only to the extent necessary to resolve the controversy'") (*quoting Vivid Techs.*, 200 F.3d at 803).

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[9] We also continue to find that "nucleic acid-lipid particle" is not limited to *in vivo* use for the same reasons that we gave in our Decision on Institution. *See* Inst. Dec. 10.

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C.   *Principles of Law*

As set forth above, we instituted trial on all of Petitioner's challenges to the claims of the '435 patent on anticipation and obviousness grounds. Dec. 18; Pet. 7. The following principles of law guide our analysis of the asserted grounds.

1. *Anticipation*

To establish anticipation, each and every element in a claim, arranged as recited in the claim, must be found in a single prior art reference. *Net MoneyIN, Inc. v. VeriSign, Inc.*, 545 F.3d 1359, 1369 (Fed. Cir. 2008); *Karsten Mfg. Corp. v. Cleveland Golf Co.*, 242 F.3d 1376, 1383 (Fed. Cir. 2001). "A reference anticipates a claim if it discloses the claimed invention 'such that a skilled artisan could take its teachings in combination with his own knowledge of the particular art and be in possession of the invention.'" *In re Graves*, 69 F.3d 1147, 1152 (Fed. Cir. 1995) (internal citation and emphasis omitted). Moreover, "it is proper to take into account not only specific teachings of the reference but also the inferences which one skilled in the art would reasonably be expected to draw therefrom." *In re Preda*, 401 F.2d 825, 826 (CCPA 1968); *see Eli Lilly & Co. v. Los Angeles Biomedical Res. Inst. at Harbor-UCLA Medical Ctr.*, 849 F.3d 1073, 1074–75 (Fed. Cir. 2017).

"Inherency is not necessarily coterminous with the knowledge of those of ordinary skill in the art. . . . Artisans of ordinary skill may not recognize the inherent characteristics or functioning of the prior art." *Atlas Powder Co. v. Ireco, Inc.*, 190 F.3d 1342, 1347 (Fed. Cir. 1999) (citing *Titanium Metals Corp. v. Banner,* 778 F.2d 775, 780, 782 (Fed. Cir. 1985).

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"[T]he discovery of a previously unappreciated property of a prior art composition, or of a scientific explanation for the prior art's functioning, does not render the old composition patentably new to the discoverer." *Id.* (citing *Titanium Metals,* 778 F.2d at 782). "It is also an elementary principle of patent law that when, as by a recitation of ranges or otherwise, a claim covers several compositions, the claim is 'anticipated' if *one* of them is in the prior art." *Titanium Metals,* 778 F.2d at 782 (emphasis added).

2. *Obviousness*

A claim is unpatentable under 35 U.S.C. § 103(a) if the differences between the claimed subject matter and the prior art are such that the subject matter, as a whole, would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398, 406 (2007). The question of obviousness is resolved on the basis of underlying factual determinations including: (1) the scope and content of the prior art; (2) any differences between the claimed subject matter and the prior art; (3) the level of ordinary skill in the art; and (4) objective evidence of nonobviousness. *Graham v. John Deere Co.*, 383 U.S. 1, 17–18 (1966). "Both the suggestion and the expectation of success must be founded in the prior art, not in the applicant's disclosure." *In re Dow Chemical Co.*, 837 F.2d 469, 473 (Fed. Cir. 1988).

In analyzing the obviousness of a combination of prior art elements, it can be important to identify a reason that would have prompted one of skill in the art "to combine . . . known elements in the fashion claimed by the patent at issue." *KSR*, 550 U.S. at 418. A precise teaching directed to the specific subject matter of a challenged claim is not necessary to establish

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obviousness. *Id.* Rather, "any need or problem known in the field of endeavor at the time of invention and addressed by the patent can provide a reason for combining the elements in the manner claimed." *Id.* at 420. Accordingly, a party who petitions the Board for a determination of unpatentability based on obviousness must show that "a skilled artisan would have been motivated to combine the teachings of the prior art references to achieve the claimed invention, and that the skilled artisan would have had a reasonable expectation of success in doing so." *In re Magnum Oil Tools Int'l, Ltd.*, 829 F.3d 1364, 1381 (Fed. Cir. 2016) (quotations and citations omitted).

In *KSR*, the Supreme Court also stated that an invention may be found obvious if trying a course of conduct would have been obvious to a person having ordinary skill:

> When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. In that instance the fact that a combination was obvious to try might show that it was obvious under § 103.

550 U.S. at 421. "*KSR* affirmed the logical inverse of this statement by stating that § 103 bars patentability unless 'the improvement is more than the predictable use of prior art elements according to their established functions.'" *In re Kubin*, 561 F.3d 1351, 1359–60 (Fed. Cir. 2009) (citing *KSR*, 550 U.S. at 417).

We analyze the asserted grounds of unpatentability in accordance with the above-stated principles. First, we address the anticipation and obviousness ground involving the '554 Publication as it is dispositive of

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many of the challenged claims. For the claims for which the '554
Publication does not either anticipate or render them obvious, we address the
remaining grounds.

D.   *Anticipation by or Obvious Over the '554 Publication*

Petitioner asserts that claims 1–20 of the '435 patent are unpatentable
as anticipated by or obvious over the '554 Publication. Pet. 51–64. With
regard to its anticipation challenge, Petitioner points to a specific
formulation taught in the '554 Publication of a nucleic acid-lipid particle
using the L054 formulation as described in Figure 16 with siRNA for
reducing HBsAg levels. *Id*. at 52. Petitioner also points to a more general
discussion in the '554 Publication which it admits "does not disclose exactly
the same ranges of lipid components from claim 1 of the '435 patent
explicitly, [but] it discloses encompassing and overlapping ranges and
specific examples falling within the claimed ranges with sufficient
specificity to anticipate." *Id.* at 51.

Patent Owner responds that the L054 formulation is not a nucleic
acid-lipid particle as set forth in the claims, and the prior art ranges are not
sufficiently specific to anticipate the challenged claims. PO Resp. 39–46.
Patent Owner also asserts that "Petitioner does not provide any showing that
the '554 Publication would have taught or suggested the use of nucleic acid–
lipid particles with high levels of cationic lipids and low levels of conjugated
lipids." *Id*. at 47.

We have reviewed the complete record before us, including the
parties' explanations and supporting evidence presented during this trial.
We determine that given the evidence on this record, Petitioner has shown

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by a preponderance of the evidence that claims 1–6, 9, 12, 14, and 15 are anticipated by the '554 Publication.

1. '554 Publication (Ex. 1004)

The '554 Publication involves lipid nanoparticles that transfect or deliver biologically active molecules, such as siRNA, to relevant cells and/or tissues in a subject to prevent, inhibit, or treat diseases, conditions, or traits in a cell, subject, or organism. Ex. 1004, Abst., ¶¶ 16–20. The '554 Publication notes that cationic lipids may be used to transport foreign nucleic acids into cells because such lipids "interact with nucleic acids through one end and lipid or membrane systems through another." *Id*. ¶ 5. The '554 Publication also identifies two structurally different complexes comprising nucleic acid and cationic lipid: a lamellar structure in which the nucleic acid monolayers sandwiched between cationic lipid bilayers, and an inverted hexagonal structure "in which nucleic acid molecules are encircled by cationic lipid in the formation of a hexagonal structure." *Id*. ¶ 13. The inverted hexagonal structure exhibits greater transfection efficiency, but has very poor stability as compared to the lamellar complex. *Id*. The '554 Publication concludes that converting the complexes to an inverted hexagonal structure using a suitable helper lipid or a co-surfactant, however, is not suitable for delivery in biological systems. *Id*.

Therefore, the '554 Publication identifies a "need to design delivery agents that are serum stable, i.e. stable in circulation, that can undergo structural transformation, for example from lamellar phase to inverse hexagonal phase, under biological conditions." *Id*. ¶ 14. In answer to this need, the '554 Publication states that:

> The present application provides compounds, compositions and methods for significantly improving the

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efficiency of systemic and local delivery of biologically active molecules. Among other things, the present application provides compounds, compositions and methods for making and using novel delivery agents that are stable in circulation and undergo structural changes under appropriate physiological conditions (e.g., pH) which increase the efficiency of delivery of biologically active molecules.

Id. ¶ 15.

The '554 Publication describes a particular embodiment, the L054 formulation. The '554 Publication states:

In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is DMOBA, the neutral lipid is distearoylphosphatidylcholine (DSPC), and the PEG conjugate is PEG-DMG. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative. This is known as formulation L053 or L054 (see Table IV).

Id. ¶ 140.

The L054 formulation was utilized in two evaluations, one of a formulated siNA composition in models of chronic HBV infection, and a second of a formulated siNA composition in an in vitro HCV replicon model of HCV infection. *See id.* ¶¶ 393, 400, 595, 603. The L054 formulation's use in the chronic HBV infection model showed an example of in vitro efficacy of siNA nanoparticles in reducing HBsAg levels in HepG2 cells. *Id.* ¶ 395. The L054 formulation's use in the in vitro HCV replicon model of HCV infection showed an "example of formulated siNA L053 and L054 (Table IV) nanoparticle constructs targeting viral replication in a Huh7 HCV replicon system in a dose dependent manner." *Id.* ¶ 400.

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The formulation for L054 is shown in Table IV of the '554

Publication as set forth below.

TABLE IV

Lipid Nanoparticle (LNP) Formulations

Formulation #	Composition	Molar Ratio
L051	CLinDMA/DSPC/Chol/PEG-n-DMG	48/40/10/2
L053	DMOBA/DSPC/Chol/PEG-n-DMG	30/20/48/2
L054	DMOBA/DSPC/Chol/PEG-n-DMG	50/20/28/2

Id. at Table IV; ¶ 92 ("In one embodiment, the molar ratio of

DMOBA:DSPC:cholesterol:PEG-DMG are 50:20:28:2 respectively, this

composition is generally referred to herein as formulation L054.").

2. Analysis

Petitioner principally relies on the L054 formulation disclosed in the

'554 Publication to establish that the challenged claims are anticipated. *See*

Pet. 51–64. We begin our analysis with the only independent claim, claim 1,

as Patent Owner's arguments focus on the limitations of this claim.

a. Claim 1

Petitioner points to the L054 formulation as disclosed in the '554

Publication as providing an example of the nucleic acid-lipid particle with

the components as set forth in claim 1 within the claimed ranges. *See* Pet.

52–56.

Dr. Janoff testifies as follows concerning this specific example.

> The '554 publication also includes various specific
> formulations, including formulation L054, which contains 50%
> cationic lipid (DMOBA), 48% non-cationic lipid (CHol/DSPC),
> and 2% conjugate lipid (PEG-n-DMG). Ex. 1004, Table 4.
> This formulation was tested, for example, with siRNA for
> reducing HBsAg levels. *See id.*, Fig. 16. The disclosed nucleic

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> acid-lipid particles meet all of the limitations in claim 1 of the
> '435 patent.

Ex. 1007 ¶ 99; *see id.* ¶¶ 144–152.

"It is also an elementary principle of patent law that when, as by a recitation of ranges or otherwise, a claim covers several compositions, the claim is 'anticipated' if *one* of them is in the prior art." *Titanium Metals,* 778 F.2d at 782 (emphasis added) (citing *In re Petering*, 301 F.2d 676, 682 (CCPA 1962)); *KSR*, 550 U.S. at 406. Here, it appears that the L054 formulation is a composition that is covered by claim 1 because it contains all of the components required by claim 1 within the claimed ranges.

Patent Owner asserts that the L054 is a lipid mixture, that may be used to make particles, but is not a particle itself. PO Resp. 39–40. Petitioner counters that the '435 patent similarly uses input formulations in describing the nucleic acid-lipid particles of the invention, which Dr. Thompson confirmed. Reply 13–14; Ex. 1019, 162:9–14.

We agree with Petitioner that the '435 patent describes nucleic acid-lipid particles in terms of mole percent of the formulation's composition, not the particle, just as in the '554 Publication. *See, e.g.,* Ex. 1001, 69:50–70:15 (Table 2 showing formulation composition in mole percent); Ex. 1004, Table IV (showing composition of L054 formulation in molar ratio); *see also* Ex. 1021 ¶ 27 (Dr. Janoff explaining that using lipid percentages in the formulations for a nucleic acid-lipid particle "was accepted practice in the field."); Ex. 1019, 162:9–14 (Dr. Thompson stating that he did not recall any description of cationic lipid analysis after particle formulation in the '435 patent). The '435 patent describes the resultant nucleic acid-lipid particle not in terms of mole percent of its components, but in terms of its size, poly-

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dispersity, and percent encapsulation of the drug. *See, e.g.,* Ex. 1001, 69:50–70:15.

    We do not agree with Patent Owner's conclusion that the final particle products of the L054 formulation all fall outside of the claimed range, and therefore, not anticipate claim 1. Dr. Thompson testifies that cholesterol-based detergents identified for use with the detergent dialysis methods described in the '554 Publication would skew the molar ratio of lipids in the finished particles relative to the starting materials because less cholesterol would be incorporated in the finished particles. Ex. 2009 ¶ 113. Dr. Thompson concludes that:

> In the L054 example, while the lipid formulation is listed as 50/20/28/2, the molar fractions of the same lipids in the resulting particle would be expected [to] be different and presumably outside the scope of the challenged claims. The L054 lipid mixture has cationic lipid content and conjugated lipid content that are at the edge of the claimed ranges. Because L054 has cationic lipid content and conjugated lipid content on the edge of the claimed range, even small differences in incorporation of components will result in lipid particles that are outside the claimed range of cationic lipid content and conjugated lipid content. For example, if cholesterol is not quantitatively incorporated, see above, particles derived from the L054 lipid mixture would have more than 2 mol % conjugated lipid.

Id. ¶ 115.

    First, the mole percentage of cationic lipid is at the low end of the range as set forth in claim 1. Therefore, any loss of the other lipid components, such as cholesterol, should result in a higher mole percentage of cationic lipid, which would be within claim 1's range of 50 mol % to 85 mol % of the total lipid present in the particle. *See* Ex. 1019:17–23

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(Dr. Thompson stating that the cationic component is the most retained during particle formation).

Second, Dr. Thompson does not definitively testify that the nucleic acid-lipid particle that is formed from the L054 formulation would fall outside of the claimed range. Although Dr. Thompson agrees that the ranges of the L054 formulation overlap with the ranges in claim 1 of the '435 patent for the required components, *see* Ex. 1020, 219:1–7, he testifies that the final formulation of the particles may be different from that of the formulation, *see id*. at 219:9–24. Dr. Thompson also testifies that in formulating the particles, he would find, as a first approximation, a bell curve for the lipid percentages of the particles, *see id*. at 220:8–221:6, and a broad distribution for the fabrication method used in the '554 Publication, *see id*. at 222:12–23.

When questioned further about this bell curve or dispersion of lipid percentages of the particles, Dr. Thompson testified as follows.

> Q: So would you have also hypothesize[d] that some of the particles in the dispersion would have greater than 50 percent cationic lipid?
> . . . .
> A: In a population of particles, there may be a X percent of particles that fall within the range. Are those the functional particles that give rise to the modest function that's reported? Who knows. The experiment was not done in a rigorous way. So it's – it's not an answerable question with any precision.
>
> You know, I can – just as the authors opine, I opine as well that the – that the particles that are being produced here are – are, have a much broader distribution than – than particles produced by other more controlled methodologies.

Id. at 224:6–21 (objection to form and foundation omitted).

With regard to whether the mole percentage of conjugated lipid would change from the formulation to the particle, Dr. Thompson discussed how

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the cholesterol content would be the most difficult to control, that losing cholesterol would cause the conjugate lipid concentration to go up, but Dr. Thompson would not confirm that none of the particles produced by the L054 formulation process would have less than 2 mole percent of conjugated lipid. *See id*. at 224:23–23. Dr. Thompson stated instead that "[t]he composition can change as a function of the formulation process." *Id*. at 226:22–23.

Based on Dr. Thompson's testimony that formulation of particles using the L054 formulation according to the processes set forth in the '554 Publication would result in a distribution of particles in terms of lipid mole percentage content, ostensibly with some particles having more cationic lipid content and less conjugated lipid content, we find Dr. Thompson's opinion that no particles formed using the L054 formulation would be within the mole percentage ranges for lipids as required by claim one is speculative, and thus, not accorded weight.

Dr. Thompson's concern about the broader range of a distribution of particles formed by the processes as described in the '554 Publication is not that no particle within the distribution would be within claim 1's required ranges, but seems to be a practical one involving a researcher's view that "usually you're not focused on those minor components of the distribution. It's the heart of the distribution that you're, that is – that's where your drug is, that's where your activity most likely lies." Dr. Thompson concludes that:

> In my analysis I was considering largely the method of formulation and the likelihood that that method of formulation would deliver the particles of specified composition, and it's my opinion that that is the wrong technique and unreliable in producing particles of a – with controlled composition.

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Id. at 228:14–20.

Anticipation, however, does not require that all of the formed particles from the L054 formulation, or even the majority of them, be within the claimed ranges as required by claim 1. Anticipation merely requires that a composition within the claimed ranges be disclosed. *See Titanium Metals*, 778 F.2d at 782. We find that the L054 formulation discloses such a composition.[10]

Patent Owner relies on its narrow definition of nucleic acid-lipid particle as requiring particles that encapsulate the nucleic acid. PO Resp. 41–43. We are not persuaded by this argument as we have stated that we do not find "nucleic acid-lipid particle" as used in the challenged claims to be so limited. *See supra* Section II.B.[11]

Because we find that claim 1 is anticipated by the L054 formulation, we decline to address Petitioner's other challenges to this claim. *See SAS Inst., Inc. v. Iancu*, 138 S. Ct. 1348, 1359 (2018) (holding that a petitioner "is entitled to a final written decision addressing all of the claims it has challenged"); *see also Beloit Corp. v. Valmet Oy*, 742 F.2d 1421, 1423 (Fed.

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[10] Patent Owner also relies on testimony from Dr. Janoff and statements in the '435 patent concerning input versus final lipid-to-drug ratios. PO Resp. 40 (citing Ex. 1001, 79:40–80:9). As Dr. Janoff testifies, however, such a change in ratio of total lipid content to drug content may be a result of one or both of those components changing from input to final product. *See* Ex. 2028, 155:1–158:14. This evidence does not establish how particular ranges for the individual lipid components from a starting formulation would change, if at all, in the final particle.

[11] We also agree with Petitioner that the '554 Publication discusses encapsulation of the nucleic acid in relation to the L054 formulation. *See* Reply 14–15; Ex. 1004 ¶¶ 11, 136, 317, 400; Ex. 1021 ¶ 28; *see also* Ex. 1004 ¶ 140 (referring to L054 formulation as a "serum-stable formulated molecular composition").

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Cir. 1984) (holding that once a dispositive issue is decided, there is no need to decide other potentially dispositive issues); *see also SZ DJI Tech. Co., LTD. v. Drone-Control, LLC*, Case IPR2018-00207, slip op. at 30–33 (Paper 44) (PTAB June 11, 2019) (discussing basis for declining consideration of other grounds when all challenged claims are shown to be unpatentable); *cf. In re Gleave*, 560 F.3d 1331, 1338 (Fed. Cir. 2009) (not reaching other grounds of unpatentability after affirming the anticipation ground).

          b. Dependent Claims

        Claims 2 through 20 depend either directly or indirectly on claim 1, which we have found to be anticipated. *See* Ex. 1001, 89:64–67, 90:54–92:22. Petitioner sets forth how these dependent claims are also either anticipated or rendered obvious by the '554 Publication supported by the testimony of Dr. Janoff. *See* Pet. 56–64; Ex. 1007 ¶¶ 153–172.

        Patent Owner responds that none of dependent claims 2–20 is anticipated or rendered obvious. *See* PO Resp. 48–52. Patent Owner specifically addresses claims 5–8, 11, and 16–20.[12]

        Although we agree with Patent Owner that the Petition regarding the patentability of the challenged dependent claims based on the '554 Publication is not a model of clarity, we find that Petitioner has sufficiently shown that dependent claims 2–6, 9, 12, 14, and 15 are anticipated by the '554 Publication. We also find, however, that Petitioner has failed to show by a preponderance of the evidence that claims 7, 8, 10, 11, 13, and 16–20 would have been either anticipated or obvious over the '554 Publication.

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[12] Patent Owner includes claim 14 in the heading for claims 16 through 20, but does not specifically discuss claim 14. *See* PO Resp. 52. Therefore, we view the inclusion of claim 14 as an inadvertent error.

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> (i)   *Anticipated Dependent Claims 2–6, 9, 12, 14 and 15*

Petitioner cites to the L054 formulation as an example teaching the
additional limitations of dependent claims 2 through 6, 9, 12, 14 and 15. *See*
Pet. 56–58, 60–62 (citing Ex. 1004 ¶¶ 395, Table IV; Ex. 1007 ¶¶ 153–157,
161, 164). For instance, claim 2 defines the nucleic acid of claim 1 as
including an interfering RNA. *See* Ex. 1001, 89:64–67. Petitioner points to
the siRNA for reducing HBsAG levels as described in Figure 16 that uses
the L054 formulation as one example of such a nucleic acid-lipid particle.
Pet. 56. For claim 3, Petitioner cites the same example as teaching that the
nucleic acid of claim 2 is further defined as a small interfering RNA or
siRNA. *See* Pet. 57.

Dependent claim 4 narrows the range for cationic lipid to 50 mol % to
65 mol % of the total lipid present in the particle. Ex. 1001, 90:58–60.
Petitioner refers to its discussion of claim 1 in which it states that "the L054
formulation tested in Figure 16 contains 50% cationic lipid (DMOBA)."
Pet. 53, 57. Dependent claim 5 defines the non-cationic lipid as a mixture of
a phospholipid and cholesterol or a derivative of them. Ex. 1001, 61–63.
Petitioner again points to the L054 formulation as an example of such a
particle. Petitioner states "[f]or example, the L054 formulation tested in
Figure 16 contains 48% non-cationic lipid (cholesterol and DSPC)." Pet. 58
(citing Ex. 1001, Table IV; Ex. 1007 ¶ 156).

Dependent claim 6 further defines the phospholipid of claim 5 as
including DSPC, which is one of the non-cationic lipids in the L054
formulation. *See* Ex. 1001, 64–67; Ex. 1004, Table IV. Petitioner also
points to an express statement in the '554 Publication that teaches suitable

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neutral lipids include the two non-cationic lipids set forth in claim 6 and mixtures of those lipids. Pet. 60 (citing Ex. 1004 ¶ 85).

Dependent claim 9 further defines the conjugated lipid as a polyethyleneglycol (PEG)-lipid conjugate. Ex. 1001, 91:7–9. Petitioner provides that "the L054 formulation tested in Figure 16 contains 2% conjugate lipid (PEG-n-DMG)." Pet. 60 (citing Ex. 1001, Table IV; Ex. 1007 ¶ 161). Claim 12 narrows that range for the conjugated lipid set forth in claim 1 to 1 mol % to 2 mol % of the total lipid present in the particle. Ex. 1001, 91:18–21. Petitioner refers to its explanation of claim 1 in which it references the L054 formulation tested in Figure 16 that contains 2% conjugate lipid (PEG-n-DMG). Pet. 55, 61–62.

Dependent claim 14 further requires a pharmaceutically acceptable carrier with the nucleic acid-lipid particle of claim 1. Ex. 1001, 92:1–3. Petitioner points to express teachings in the '554 Publication that the "pharmaceutical carrier is generally added following formulated siNA composition formation. Thus, after the formulated siNA composition is formed, the formulated siNA composition can be diluted into pharmaceutically acceptable carriers such as normal saline." Pet. 62 (quoting Ex. 1004 ¶ 502; Ex. 1007 ¶ 166).

Dependent claim 15 further requires contacting a cell with the nucleic acid-lipid particle of claim 1 to introduce the nucleic acid into the cell. Ex. 1001, 92:4–7. Petitioner states that this additional limitation is met in part by referring back to its discussion of claim 1. With reference to the nucleic acid-lipid particles of claim 1, Petitioner states that "[o]ne example of such particles with siRNA for reducing HBsAg levels using the L054 formulation are described in Figure 16," and quotes the '554 Publication as stating, "FIG. 16 shows a non-limiting example of in vitro efficacy of siNA

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nanoparticles in reducing NBsAg levels in Hep G2 cells . . . treated with formulated active siNA L053 and L054 nanoparticles . . . ." Pet. 52.

We find that Petitioner has shown that the L054 formulation anticipates claims 2 through 6, 9, 12, 14, and 15 by the L054 formulation and its use in experiments as referenced above as taught in the '554 Publication. As set forth by Petitioner, the L054 formulation not only meets the limitations of claim 1, but also the additional limitations of dependent claims 2 through 6, 9, and 12 arranged as in the claim as set forth above. Also, the '554 Publication expressly states that formulated siNA compositions, such as L054, may be diluted into a pharmaceutical carrier such as saline, thus anticipating claim 14. Also, the use of the L054 nanoparticles to show in vitro efficacy of siNA nanoparticles in reducing HBsAg levels in HepG2 cells anticipates claim 15.

Patent Owner specifically responds to Petitioner's arguments concerning claims 5 and 6. PO Resp. 48–49. Patent Owner states, with respect to claim 5, it cannot be anticipated because the L054 formulation does not anticipate claim 1. *Id*. at 48. Because we have found that claim 1 is anticipated by the L054 formulation, this argument is not persuasive. Patent Owner's argument concerning claim 6 refutes an obviousness analysis. Because we find that claim 6 is anticipated by the L054 formulation, we also are not persuaded by this argument.

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(ii)   *Dependent Claims 7, 8, 10, 11, 13, and 16–20 are not Unpatentable Over the '554 Publication*

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We agree with Patent Owner that Petitioner has failed to establish that dependent claims 7, 8, 10, 11, 13, and 16–20 are unpatentable. Petitioner offers an anticipation challenge to claims 7, 8, 10, 13, and 16–20 based on the teachings of the '554 Publication. Pet. 58–64. Petitioner also challenges

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claims 7, 8, and 11 based on obviousness over the '554 Publication. *Id.* at 58–62. Finally, Petitioner sets forth an obviousness challenge to claims 7, 8, 10, 11, 13, and 16–20 based on the teachings of the '196 PCT and the '189 Publication. Pet. 42–48. We determine that Petitioner has not shown by a preponderance of the evidence on any of these challenges that these dependent claims are unpatentable.

Although we did not need to reach the following arguments in determining the unpatentability of claim 1, we will reach these arguments here as they relate to the dependent claims. *See* Pet. 52–56. The '554 Publication discloses several different ranges for each lipid component, some of which touch or overlap the claimed ranges, some of which do not. *See* Ex. 1004 ¶¶ 116–118, 313. As Patent Owner states, Petitioner cites these different ranges for the different lipid components of claim 1 as set forth in paragraphs 116, 118, and 313 of the '554 Publication without demonstrating how all of these ranges for the different components relate to each other with sufficient specificity to anticipate claim 1. *See* Pet. 52–55; PO Resp. 44–45. Therefore, we find that claim 1, that recites ranges of each lipid component, is not anticipated by the teaching of these ranges. *See Atofina v. Great Lakes Chem. Corp*, 441 F.3d 991, 999 (Fed Cir. 2006).

In sum, the only basis on which we conclude that claim 1, or any claim that depends on claim 1, is anticipated is by the L054 formulation that has lipid components in the claimed ranges for claim 1. This informs our discussion of the remaining dependent claims set forth below.

Alleged Anticipation of Dependent Claims 7 and 8

For dependent claims 7 and 8, which provide specific ranges for the phospholipid and cholesterol components of the nucleic acid-lipid particle, respectively, claim 7, which depends from claim 5, requires the phospholipid

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to be 3–15 mol % of the total lipid present. Ex. 1001, 91:1–3. Claim 8, which also depends from claim 5, requires the cholesterol to be from 30–40 mol % of the total lipid present in the particle. *Id.* at 4–6. Petitioner sets forth an anticipation and obviousness challenge from the teachings of the '554 Publication for each of claims 7 and 8. *See* Pet. 58–60.

For claim 7, Petitioner points to a range for the total amount of neutral lipid component, 20–85 mol %, as compared to a range for the cholesterol component of 20 to 45 mol %, when it is present, and concludes a range for phospholipid is 0–40 mol %. Petitioner states that

> [n]ot only does the disclosed range encompass the claimed range, when combined with a cationic lipid proportion in the 60% range and cholesterol in the 20–40% range, the range for the phospholipid is decreased to 0%-20%. Given the breadth of the claimed range for the phospholipid, these disclosures are sufficiently specific to anticipate the claimed range.

Pet. 58–59 (citing Ex. 1007 ¶ 158).

Patent Owner responds that no range for phospholipid is disclosed in the '554 Publication. PO Resp. 49.

We agree with Patent Owner that Petitioner fails to show that claim 7 is anticipated by the teachings of the '554 Publication. As Patent Owner points out, Petitioner points to no affirmative teaching in the '554 Publication of a specific range for the amount of phospholipid in the nucleic acid-lipid particle. *See* Pet. 58–59. Petitioner makes some assumptions about the various other components in the particle, and then calculates a range for the phospholipid that encompasses the claimed range based on those assumptions and assuming that the phospholipid makes up the balance of the non-cationic lipid in the particle. *Id.* Petitioner makes no mention of the conjugated lipid component that is also required by the claim or why one

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of skill in the art would choose 60 mol % for the cationic lipid component.
Id. We determine that Petitioner has failed to show that the claimed range
for the phospholipid is taught by the '554 Publication. *See Atofina v. Great
Lakes Chem. Corp*, 441 F.3d 991, 999 (Fed Cir. 2006). Therefore, we
determine that Petitioner has failed to show that claim 7 is anticipated by the
teachings of the '554 Publication.

For claim 8, Petitioner cites to a range for cholesterol, from 20 to
45 % of the total lipid present, which encompasses the claimed range, and
also references a specific formulation that has an amount of cholesterol
within the claimed range. Pet. 59–60. Petitioner concludes that "[g]iven the
breadth of the claimed range, these disclosures are sufficiently specific to
anticipate the claimed range." Pet. 60 (citing Ex. 1007 ¶ 160).

Patent Owner responds that Petitioner has offered no relationship
between the disclosure mapped for claim 8 to that mapped for claims 1 and 5
from which claim 8 depends. PO Resp. 49. We agree. As we stated above,
Petitioner offers no particular embodiment with overlapping ranges for each
component of the nucleic acid-lipid particle for claim 8 that includes the
limitations of claims 1 and 5 from which claim 8 depends. Therefore, we
determine that Petitioner has failed to show that claim 8 is anticipated by the
teachings of the '554 publication.

Alleged Anticipation of Dependent Claim 10

Claim 10 adds the requirement that the PEG-lipid conjugate comprise
a PEG-DAG conjugate, a PEG-DAA conjugate, or a mixture of these.
Ex. 1001, 91:10–13. Petitioner points to a disclosure in the '554 publication
of PEG-DAG conjugates and states that "[b]ecause one of the listed species
of PEG-lipid conjugates is disclosed, this element is anticipated." Pet. 60–
61 (citing Ex. 1004 ¶ 86; Ex. 1007 ¶ 162).

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Similar to claim 8, Petitioner fails to show any relationship between the disclosure of the PEG-DAG conjugate that teaches the additional requirement for claim 10 to any disclosure that teaches the limitations of claims 1 and 9 from which claim 10 depends. For example, paragraph 86 of the '554 publication cited by Petitioner refers to suitable PEG-DAG conjugates, but does not indicate that the PEG-lipid conjugate in the claimed combination may be the described PEG-DAG conjugates. Therefore, we determine that Petitioner has failed to show that claim 10 is anticipated by the teachings of the '554 Publication.

Alleged Anticipation of Dependent Claim 13

Dependent claim 13 requires the nucleic acid to be fully encapsulated in the nucleic acid lipid particle of claim 1. Ex. 1001, 91:22–24. Petitioner asserts that the '554 Publication teaches that "encapsulation of anionic compounds using cationic lipids is essentially quantitative due to electrostatic interaction." Pet. 62 (quoting Ex. 1004 ¶ 11). From this teaching, Dr. Janoff concludes that a person or ordinary skill would understand that full encapsulation only requires "an excess of cationic lipid with regard to the nucleic acid for electrostatic interaction." *Id*. (citing Ex. 1007 ¶ 165).

It is not readily apparent, however, from the L054 formulation and the discussion concerning this formulation that the nucleic acid lipid particle is fully encapsulated. *See* Ex. 1004 ¶¶ 87, 89, 92, 140, 395, 400, 595, 603. Although the L054 formulation is said to be serum stable, *see* Ex. 1004 ¶ 140, there is no mention of whether the nucleic acid is fully encapsulated within the lipid particle. *See* Ex. 1004 ¶¶ 87, 89, 92, 140, 395, 400, 595, 603. Other examples described in the '554 Publication do expressly describe encapsulation of the nucleic acid in the lipid particle, although

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without identifying the extent of the encapsulation. *See, e.g.,* Ex. 1004 ¶¶ 397 (describing L051 nanoparticle "encapsulated active siNA molecules), 398 (same), 408 (same for L077, L069, L080, L082, L083, L060, L061, and L051). Also, Dr. Janoff does not discuss what would be considered an excess of cationic lipid as compared to the amount of nucleic acid that would yield a nucleic acid-lipid particle that fully encapsulates the nucleic acid or whether the L054 formulation would provide such an excess of cationic lipid. *See* Ex. 1007 ¶ 165.

For these reasons, we determine that Petitioner has not shown by a preponderance of the evidence that claim 13 is anticipated by the '554 Publication.

Alleged Anticipation of Dependent Claims 16–20

Claims 16–20 require *in vivo* delivery or administration of a nucleic acid-lipid particle of claim 1 to a mammalian subject. *See* Ex. 1001, 92:8–23. Petitioner points to several disparate paragraphs of the '554 Publication as teaching the additional limitations of claims 16–20 relating to the *in vivo* delivery or administration of the claimed lipid particle to a mammalian subject to treat a disease or disorder such as a viral infection, liver disease, or cancer. *See* Pet. 63–64.

For instance for claim 16, Petitioner points to the definition of "subject" in the '554 Publication, which includes a mammal or mammalian cells, to establish that such *in vivo* delivery or administration to a mammalian subject of claim 1's nucleic acid-lipid particle is taught. *See* Pet. 63 (citing Ex. 1007 ¶ 168). Petitioner also points to particular embodiments to establish the additional limitations relating to *in vivo* delivery and administration as taught in claims 17–20. *See* Pet. 63–64 (citing Ex. 1004 ¶¶ 21, 274, 275, 310). None of these embodiments,

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however, relates to the L054 formulation, which we have found anticipates claim 1's nucleic acid lipid particle.

The '554 Publication does not teach that every formulation described for a lipid particle is suitable for *in vivo* delivery or administration to a mammalian subject. For instance, the '554 Publication describes its invention as featuring

> compositions, and methods of use for the *study, diagnosis*, and treatment of traits, diseases and conditions that respond to the modulation of gene expression and/or activity in a subject or organism. . . . Such novel cationic lipids, microparticles, nanoparticles and transfection agents are useful, for example, in providing compositions to prevent, inhibit, or treat diseases, conditions, or traits *in a cell*, subject or organism.

Ex. 1004, Abst. (emphasis added).

The L054 formulation is only shown to be used *in vitro* to treat cells, *id*. ¶¶ 395, 400, 595, 603, and Petitioner does not point to any teaching indicating that the L054 formulation may be appropriate for systemic use. Therefore, we determine that Petitioner has failed to show that claims 16–20 are anticipated by the '554 Publication.

Alleged Obviousness of Dependent Claims 7, 8, and 11
over the '554 Publication

For claims 7 and 8, Petitioner relies on *In re Peterson*, 315 F.3d 1325 (Fed. Cir. 2003) to establish even a slight overlap in ranges renders them obvious. *See* Pet. 58–60. As we discussed previously for claim 7, Petitioner makes assumptions concerning the disclosed ranges for the other components of a lipid particle, but can point to no particular range for the phospholipid that overlaps the range required by claim 7. *Id*. at 59; PO Resp. 49; Ex.2009 ¶¶ 148–150. Also, Petitioner has not shown how achieving the claimed range for the phospholipid would be accomplished by

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mere optimization or routine experimentation. *Id.*; *Peterson*, 315 F.3d at

1330.

Petitioner points to disclosure in the '554 Publication that

encompasses the claimed range for cholesterol as required by claims 8, and

points to a particular example that has the amount of cholesterol within the

claimed range without referring to any other of the required components of

the lipid particle or ranges for those components. Pet. 59–60; PO Resp. 49–

50; Ex. 2009 ¶¶ 151–155. Again, Petitioner has not shown how achieving

the claimed range for cholesterol as required by claim 8 would be

accomplished by mere optimization or routine experimentation. *Id.*

Challenged claim 11 depends from claim 10, which we found was no

anticipated by the '554 Publication. For Petitioner's obviousness challenge

based on the '554 Publication, it asserts that the PEG-DAA conjugates

required by claim 11 "could be used in lieu of . . . PEG-DAG

conjugates . . ." required by claim 10. Pet. 61. Patent Owner points out that

the '554 Publication does not disclose PEG-DAA conjugates at all, and

questions the interchangeability of PEG-DAG and PEG-DAA conjugates.

PO Resp. 50-51. Because we did not find claim 10 to be anticipated, and

Petitioner makes no obviousness challenge to claim 10 based on the '554

Publication, we find Petitioner's argument concerning the obviousness of

claim 11 to be unpersuasive.

We determine that Petitioner has failed to establish by a

preponderance of the evidence that claims 7, 8, or 11 would have been

obvious over the '554 publication.

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Alleged Obviousness of Dependent Claims 7, 8, 10, 11, 13, and 16–20
over the '196 PCT and the '189 publication

Petitioner's obviousness challenge for dependent claims 7, 8, 10, 11, 13, and 16–20 under ground 1 fairs no better. *See* Pet. 42–48.[13] For these dependent claims, Petitioner states where the limitation added by the dependent claim is taught in the asserted references, but does not address the relationship between the different ranges for the components of the particles or any reason why one of skill in the art would combine these teachings with those that allegedly taught the limitations of the claims from which the claim at issue depends. *See id.*; PO Resp. 14–32. Petitioner also does not address whether one of skill in the art would have a reasonable expectation of success in making the claimed combination. Pet. 42–48; PO Resp. 14–32. For none of these claims does Petitioner discuss how the subject matter of each claims as a whole would have been obvious in light of the teachings of the asserted art. *Id.*; PO Resp. 14–15 (stating that petition fails to acknowledge that concentrations of different lipid components are highly interdependent).

We find that Petitioner has not shown by a preponderance of the evidence that claims 7, 8, 10, 11, 13, or 16–20 are unpatentable would have been obvious over the '196 PCT and the '189 Publication.

E.   Objections and Other Arguments

Patent Owner also filed a Notice of Objection to Petitioner's Demonstrative Exhibits. Paper 48. Patent Owner's objections are presented

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[13] Claims 1 and 4 are the only two claims specifically addressed in ground 2. Because we have determined that these claims are anticipated, we need not address this ground here. Pet. 48–51.

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as general categories of objections with identification of Petitioner's slides

that it contends fall within those broad categories. *Id.* at 1. However, the

Board will not sort through multiple slides in an effort to discern what

particular information is subject to Patent Owner's general objection.

Nevertheless, we overrule Patent Owner's objections to Petitioner's

demonstrative exhibits (slides), at least because demonstrative exhibits are

not evidence, and we rely on evidence in the trial record in reaching our

Decision. *See* Office Patent Trial Practice Guide, August 2018 Update, 21,

83 Fed. Reg. 39,989 (Aug. 13, 2018) ("Demonstrative exhibits used at the

final hearing are aids to oral argument and not evidence").

Patent Owner asserts that the testimony of Dr. Janoff should be

accorded no weight. *See* PO Resp. 4–8. Patent Owner argues that,

according to Dr. Janoff's testimony, his declaration is "based on the

petition," and thus constitutes attorney argument. *Id.* at 4–5 (citing Ex. 1007

¶¶ 5–7, 27; Ex. 2028, 26:12–27:5, 91:18–92:20, 92:21–93:11). Patent

Owner argues further about the conduct of Dr. Janoff and Petitioner's

counsel during cross-examination, contending that it was "disruptive and

prejudicial," and that "[s]uch conduct is particularly prejudicial in the

context of the present proceeding, as Patent Owner has repeatedly raised

issues as to the lack of clarity in the petition materials." *Id.* at 6–7 (citing

multiple excerpts from Ex. 2028).

Petitioner responds that Dr. Janoff testified that "he was aware of the

Petition when executing his declaration," and also "testified unequivocally

that the opinions in his declaration are his opinions in the matter." Reply 24

(citing Ex. 2028, 89:4–92:20, 26:12–18). Petitioner further responds that

"[t]he transcript illustrates that counsel for Patent Owner repeatedly asked

questions devoid of any context and without reference to either Dr. Janoff's

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declaration or the '127 patent," but that "Dr. Janoff, nonetheless, provided answers to the extent possible." *Id.* at 24–25 (citing multiple excerpts from Ex. 2028).

We decline to exercise the extraordinary remedy of according "no weight" to Dr. Janoff's testimony based on the contention that Dr. Janoff's testimony is attorney argument because it is based on the Petition. PO Resp. 4–8. Similarly, although Dr. Janoff may not have been as responsive to some questions during his deposition as may be appropriate, we decline to accord "no weight" to his entire testimony (*see* id.), particularly given that some questions appear vague, or lacking context or proper foundation. *See, e.g.,* Ex. 2028, 35:7–8, 29:19–20, 56:24–57:17; *see also* Reply 24–25.

In making the argument that "Dr. Janoff's declaration merely adopts the attorney arguments set forth in the petition," Patent Owner omits the remainder of the cited quotes from Dr. Janoff's declaration. *See* PO Resp. 4–5. All of those quotes actually state that Dr. Janoff's opinions are based on the Petition "and other documents and materials identified in this declaration" (Ex. 1007 ¶ 26) or "and the exhibits cited in the Petition as well as other documents" (*id.* ¶¶ 5–7).

F.   *Patent Owner's Motion to Amend*

Patent Owner's contingent Motion to Amend proposes to substitute claims 21–40 for issued claims 1–20, if the latter are determined to be unpatentable. We have determined that Petitioner has shown by a preponderance of the evidence that the challenged claims 1–6, 9, 12, 14, and 15 are unpatentable as anticipated by the teachings of the '554 Publication.

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Thus, we consider Patent Owner's contingent Motion to Amend concerning substitute claims for these claims.

Proposed substitute independent claim 21 is representative and is reproduced below, with additional matter being underlined and deleted material marked with double-brackets. Patent Owner proposes amending dependent claims 2–20 to reflect dependencies relative to original claims 2–20 in light of new substitute claim 21. *See* Mot. 8. According to Patent Owner, "[n]o further substantive amendments are proposed for these claims." Mot. 8.

> 21. (Substitute for claim 1) A <u>serum-stable</u> nucleic acid-lipid particle comprising:
> (a) a nucleic acid;
> (b) a cationic lipid comprising from 50 mol % to [[85]] <u>75</u> mol % of the total lipid present in the particle;
> (c) a non-cationic lipid comprising from [[13]] <u>23</u> mol % to 49.5 mol% of the total lipid present in the particle; and
> (d) a conjugated lipid that inhibits aggregation of particles comprising from 0.5 mol % to 2 mol % of the total lipid present in the particle;
> <u>wherein the particle is formulated such that the nucleic acid is not substantially degraded after exposure of the particle to a nuclease at 37ºC for 20 minutes.</u>

Id. at 3–4.

1. *Procedural Requirements for a Motion to Amend*

In an *inter partes* review, amended claims are not added to a patent as of right, but rather must be proposed as a part of a motion to amend. 35 U.S.C. § 316(d). "During an *inter partes* review instituted under this chapter, the patent owner may file 1 motion to amend the patent," and "[f]or

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each challenged claim, propose a reasonable number of substitute claims."
Id.; *see also* 37 C.F.R. § 42.121(a)(3).

Patent Owner's proposed substitute claims, however, must meet the statutory requirements of 35 U.S.C. § 316(d) and the procedural requirements of 37 C.F.R. § 42.121. *See Lectrosonics, Inc. v. Zaxcom, Inc.*, Case IPR2018-01129, slip op. at 2 (PTAB Feb. 25, 2019) (Paper 15) (precedential); Memorandum "Guidance on Motions to Amend in view of *Aqua Products*" (Nov. 21, 2017) (https://www.uspto.gov/sites/default/files/documents/guidance_on_motions_ to_amend_11_2017.pdf) ("Board's Memorandum"). Although a motion to amend must comply with these requirements, a patent owner does not bear a burden to prove its proposed claims are patentable. *Aqua Prods., Inc. v. Matal*, 872 F.3d 1290, 1296 (Fed. Cir. 2017) (en banc) (stating Board may not place burden of persuasion on the patent owner).

Accordingly, Patent Owner must demonstrate: (1) the amendment proposes a reasonable number of substitute claims; (2) the amendment responds to a ground of unpatentability involved in the trial; and (3) the amendment does not seek to enlarge the scope of the claims of the patent or introduce new subject matter, such that the proposed substitute claims are supported in the original disclosure. *See* 35 U.S.C. § 316(d); 37 C.F.R. § 42.121. We determine that Patent Owner's Motion to Amend satisfies these procedural requirements for the reasons set forth below.

   a. *The Amendment Proposes a Reasonable Number*
      of Substitute Claims

We find that Patent Owner proposes a reasonable number of substitute claims. Patent Owner proposes one substitute claim for each challenged claim, which is a presumptively reasonable number of substitute claims. *See*

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37 C.F.R. § 42.121(a)(3) ("The presumption is that only one substitute claim would be needed to replace each challenged claim.").

> b. *The Amendment Responds to a Ground of Unpatentability Involved in the Trial*

We find that Patent Owner's proposed substitute claims specifically respond to the unpatentability grounds set forth in the Petition. In two of the obviousness grounds involved in the *inter partes* review, Petitioner relies upon the teachings in the '196 PCT and the '189 Publication of nucleic acid-lipid particles that comprise a nucleic acid, and the lipid components—a cationic lipid, a non-cationic lipid, and a conjugated lipid—in mol percentage ranges that Petitioner asserts overlap with the claimed ranges. *See* Pet. 32–48; *see* Ex. 1007 ¶¶ 106–118. In the third obviousness ground, the only ground that includes an anticipation challenge, Petitioner relies upon the teachings in the '554 Publication of "encompassing and overlapping ranges and specific examples falling within the claimed ranges with sufficient specificity to anticipate." Pet. 51 (citing Ex. 1007 ¶ 143).

In response to Petitioner's challenges, Patent Owner contends that "substitute claim 21 amends independent claim 1 by reciting a narrower range for the concentration of the cationic lipid, and a narrower range for the concentration of non-cationic lipid." Mot. 3. Patent Owner additionally contends that addition of the term "serum stable" to the preamble of substitute claim 21 and addition of the phrase "wherein the particle is formulated such that the nucleic acid is not substantially degraded after exposure of the particle to a nuclease at 37ºC for 20 minutes" respond to the instituted grounds. *Id.*

Petitioner argues that the proposed substitute claims do "not respond to a ground of unpatentability involved in the trial" because the mol %

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ranges for cationic lipid in the proposed substitute claims also overlap with the ranges taught by the cited prior art references. Opp. 2 (citing 37 C.F.R. § 42.121(a)(2)(i)). Petitioner contends that Patent Owner proposed narrower ranges because "it is apparently geared toward more closely aligning the claims with testing that the Patent Owner relies upon as evidence of unexpected results." *Id.* (citing Mot. 16). Petitioner also argues that proposed substitute claims 22–40 do not respond to a ground of unpatentability involved in the trial because "Patent Owner does not propose changes to dependent claims 2–20" and "[t]he proposed amendments do nothing to address that the additional limitations present in these claims are disclosed in the prior art." *Id.* at 1 n.1.

Patent Owner replies that the proposed substitute claims respond to the grounds of unpatentability because they relate to serum stability and nuclease resistance, both of which are requirements for systemic use of the claimed particles. Reply Opp. 3. Patent Owner also explains that the proposed substitute cationic lipid range of "50 mol % to 75 mol %" is outside of the range disclosed in the prior art '189 Publication, and "Petitioner does not provide a reason to increase the amount of cationic lipid." *Id.* at 3–4.

Patent Owner further contends that the proposed substitute claims are patentable over the '554 Publication because the reference discloses the use of chemically-modified RNA constructs to protect the nucleic acid from enzymatic degradation, rather than the '435 patent's protection mechanism of encapsulating the nucleic acid within the lipid portion of the particle. *Id.* (citing Ex. 1001, 22:55–62). Patent Owner also argues that the overlapping ranges "may invoke a rebuttable presumption of obviousness under the specific rationale of 'routine optimization'" but "Petitioner offers no

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argument or evidence demonstrating that arriving at the claimed ranges of the lipid components was routine." *Id.* at 7–8.

Because Petitioner argues in part that the challenged claims are unpatentable because the claimed ranges of lipids overlap with the ranges taught by the prior art references, and Patent Owner attempts to address these challenges by narrowing the claimed ranges, we find that the amendment responds to a ground of unpatentability involved in the trial – the merits of which we will discuss below.

c. *The Amendment Does Not Seek to Enlarge the Scope of the Claims of the Patent or Introduce New Subject Matter*

We find that the written description of the '435 patent provides adequate support for the proposed substitute claims. The '435 patent claims priority to Provisional Application No. 61/045,228, filed April 15, 2008 ("the '228 provisional," Ex. 2041) "through a series of three continuation applications: U.S. Application No. 13/928,309 filed June 26, 2013, ('the '309 application,' EX2044); U.S. Application No. 13/253,917 filed October 5, 2011, ('the '917 application,' EX2043); and U.S. Application No. 12/424,367 filed April 15, 2009 ('the '367 application," EX2042)." Mot. 4.

Petitioner asserts that the proposed substitute claims "lack written description support and an enabling disclosure for the different nucleic acid payloads recited therein." Opp. 1. Petitioner contends, "the substitute claims broadly cover any nucleic acid payload—despite wide variations in potential nucleic acids and without support for anything but siRNA" and represent "Patent Owner's attempt to extend its disclosures to cover other nucleic acid payloads" because "Petitioner is an mRNA company, while Patent Owner (and its predecessors) have traditionally focused on siRNA." *Id.* at 2–3.

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According to Petitioner, "Patent Owner points to testing of only siRNA payloads with a limited number of exemplar lipid components and with limited formulation processes" and admits "that changes to the payload, identity of lipid components, or production techniques can all impact the particle properties and resulting efficacy" and "the test data did not show the claimed formulations outperforming the prior art 2:40 formulation." *Id.* at 9–10 (citing Ex. 1022 ¶ 63; Ex. 1019; Ex. 1020). Petitioner explains, the '435 patent's "Background of the Invention" section discusses using siRNA for gene silencing and states that "the purpose of the invention is 'downregulating the expression of genes of interest to treat or prevent diseases and disorders such as cancer and atherosclerosis," but "[t]his is not a function of mRNA." *Id.* at 12–13 (citing Exs. 1001, 1:39–51; 1022 ¶ 70). Petitioner also explains that the '435 patent's specification identifies the nucleic acids in SNALPs as "one or more interfering RNA molecules such as siRNA, aiRNA, and/or miRNA" and "the preferred embodiments are described as having an siRNA payload: 'in preferred embodiments, the active agent or therapeutic agent comprises an siRNA.'" *Id.* (quoting Ex. 1001, 3:32–37, 14:62–17:47).

Patent Owner responds that its Motion to Amend and the original disclosure provide written description support for the use of "'nucleic acid' including mRNA." Reply Opp. 9. Specifically, Patent Owner's Motion to Amend discusses several disclosures in the '228 provisional that provide written description support for the nucleic acid limitation in challenged claim 1 and proposed substitute claim 21. Mot. 6 (citing Exs. 2041 ¶¶ 10, 19, 25–29; 2045 ¶¶ 17–18, 61, 76, 140, 307; 2040 ¶ 28). The disclosures highlighted by Patent Owner discuss encapsulation of "a nucleic acid" or an interfering RNA and merely provide siRNA as an example of a nucleic acid

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by stating "(*e.g.*, the siRNA molecule)." *See* Ex. 2041 ¶¶ 10, 25–29.
According to Patent Owner, "[t]he disclosure is directed to 'stable nucleic
acid-lipid particles encapsulating a nucleic acid.'" Mot. 6 (citing Exs. 2041
¶ 10; EX2045 ¶¶ 289, 329, 331; 2040 ¶ 28).

Patent Owner further contends that the '435 patent's specification
provides support for the use of mRNA as a nucleic acid. Reply Opp. 9
(citing Ex. 1001 10:26–35). In fact, the specification states that "[t]he term
'nucleic acid' as used herein refers to a polymer containing at least two
deoxyribonucleotides or ribonucleotides in either single- or double-stranded
form and includes DNA and RNA" and further explains that "RNA may be
in the form of siRNA, asymmetrical interfering RNA (aiRNA), microRNA
(miRNA), mRNA, tRNA, rRNA, tRNA, viral RNA (vRNA), and
combinations thereof." Ex. 1001, 10:26–36.

We find Patent Owner's examples of written description support for
the nucleic acid limitation, especially as set forth in the Specification of the
'435 patent, sufficient. The '435 patent's specification discusses nucleic
acids broadly, but also provides examples of nucleic acids, including
mRNA. Ex. 1001, 10:26–36. Thus, we find Patent Owner's proposed
substitute claims satisfy the written description requirement.

2. *Patentability Analysis of Proposed Substitute Claims*

In accordance with *Aqua Products*, Patent Owner does not bear the
burden of persuasion to demonstrate the patentability of the substitute claims
presented in the Motion to Amend. Rather, ordinarily, "the petitioner bears
the burden of proving that the proposed amended claims are unpatentable by
a preponderance of the evidence." *Bosch Auto. Serv. Sols., LLC v. Matal*,
878 F.3d 1027, 1040 (Fed. Cir. 2017), *as amended on reh'g in part* (Mar.

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15, 2018). The Board itself also may justify any finding of unpatentability

by reference to evidence of record in the proceeding. *Id.* (citing *Aqua*

Products, 872 F.3d at 1311 (O'Malley, J.)). Thus, the Board determines

whether the proposed substitute claims are unpatentable based on the

entirety of the record, including any argument made by Petitioner.

In its Opposition, Petitioner asserts that the proposed substitute claims

do not remedy the unpatentability issues. Opp. 1. According to Petitioner,

the proposed substitute claims are unpatentable under 35 U.S.C. § 103 as

obvious over 1) the '196 PCT and the '189 Publication; and 2) the '196

PCT, the '189 Publication, Lin, and Ahmad; under 35 U.S.C. §§ 102 and

103 as anticipated by and obvious over the '554 Publication; and under

35 U.S.C. § 112 for lack of written description support and enablement. *Id.*

at 8–20. We will focus our discussion on anticipation by the '554

Publication as we find this dispositive of the motion.

Petitioner contends that Patent Owner's addition of "serum-stable" to

the preamble of claim 1 is non-limiting because preambles are generally

considered to be non-limiting. *Id.* at 1, 3–4 (citing *Catalina Marketing Int'l,*

Inc. v. CoolSavings.com, Inc., 289 F.3d 801, 808 (Fed. Cir. 2002)).

According to Petitioner, a POSITA would not "consider the term 'serum-

stable' limiting in the claims." *Id.* (citing Ex. 1022 ¶ 53).

Petitioner further argues that even if the preamble were considered

limiting, "the cited prior art references disclose serum-stable particles at

greater than 50 mol% cationic lipid" and "[e]ach of the three primary

references disclose the desire for serum-stable particles." *Id.* at 4 (citing

Ex. 1002 ¶¶ 2, 15–16, 120, 134; Ex. 1003, ¶¶ 182, 191, 217; Ex. 1004 ¶¶ 14–

15, 158; Ex. 1022 ¶ 54). Petitioner points to disclosures in the '189

Publication and the '554 Publication that discuss a series of *in vivo*

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experiments with formulations matching the cationic lipid ranges in the

proposed substitute claims. *Id.* (citing Ex. 1003 ¶¶ 351–391; Ex. 1004

¶ 408, Table IV, Fig. 29). Petitioner concludes "[a] POSITA would

understand these disclosures in the context of the prior art references to

disclose serum-stable particles at greater than 50 mol% cationic lipid." *Id.*

(citing Ex. 1022 ¶ 54).

Petitioner additionally contends that the limitation "wherein the

particle is formulated such that the nucleic acid is not substantially degraded

after exposure of the particle to a nuclease at 37ºC for 20 minutes" fails to

"differentiate over the prior art, given that serum-stable particles that resist

nuclease degradation are disclosed in the prior art already of record." *Id.* at

1–2. According to Petitioner, the limitation "does not require the particle to

be 'serum stable' as such resistance can be tested *in vitro* using a nuclease."

Id. at 3 (citing Ex. 1020, 159). In support of this statement, Petitioner points

to *in vitro* testing of the L054 formulation in the '554 Publication. *Id.* at 6

(citing Ex. 1004, Table IV). Petitioner explains that the L054 formulation

(tested *in vitro*) and the L060 formulation (tested *in vivo*) both contained the

cationic lipid, DMOBA, and the L060 formulation showed *in vivo* efficacy

with 52 mol % DMOBA. *Id.* (citing Ex. 1004 Table IV, ¶ 408, Fig. 29; Ex.

1022 ¶ 59). Petitioner also argues that "[e]ach of the three primary

references disclose nucleic acid-lipid particles that can also withstand

nuclease exposure and Patent Owner's prior disclosures disclose these exact

parameters." *Id.* at 7–8 (citing Ex. 1002; Ex. 1003).

Patent Owner responds that the addition of "serum-stable" to the

preamble of claim 1 "reinforces that the claims require a particle in which

the nucleic acid is encapsulated in the particle so as to protect the nucleic

acid from enzymatic degradation" and "is tied to the added limitation of

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'wherein the particle is formulated such that the nucleic acid is not substantially degraded after exposure of the particle to a nuclease at 37ºC for 20 minutes.'" *Id.* Patent Owner also contends that Petitioner's argument that the '554 Publication discloses "in vivo efficacy of a particle having 52 mol% DMOBA . . . is irrelevant as toxicity was never assessed in the '554 publication." *Id.* at 5–6.

After considering the parties' arguments and the remainder of the record, we conclude that the proposed substitute claims do not overcome the prior art references and find them unpatentable for the same reasons as discussed above.

For purposes of this analysis, we assume that "serum stable" is limiting. The L054 formulation is also within the more narrow ranges required by substitute claim 21. *Compare* Ex. 1004 ¶ 92, *with* substitute claim 21. The question becomes whether the addition of serum stability sufficient to meet the parameters of the wherein clause is sufficient to distinguish the substitute claims from the prior art.

The L054 formulation is described as serum stable. *Id.* ¶ 140. In Example 9, titled "Evaluation of Formulated siNA Compositions in Models of Chronic HBV Infection," an in vitro analysis of the activity of nanoparticle formulation L054 was performed. *Id.* ¶ 595. The '554 Publication describes adding the siNA nanoparticle formulation to wells containing HepG2 cells with media. *Id.* "The cells were incubated for 4 days, the media was then removed, and assayed for HBsAg levels. . . . FIG. 16 shows level of HBsAg from formulation (Formulations L053 and L054, Table IV) active siNA treated cells compared to untreated or negative control treated cells." *Id.*

The '554 Publication concludes that:

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> In these studies, a dose dependent reduction in HBsAg levels
> was observed in the active formulated siNA treated cells using
> nanoparticle formulations L051, L053, and L054, while no
> reduction is observed in the negative control treated cells. This
> result indicates that the formulated siNA compositions are able
> to enter the cells, and effectively engage the cellular RNAi
> machinery to inhibit viral gene expression.

Id. In describing this Example, the '554 Publication states that incubation
was carried out at 37º C. *Id*.

The discussion of this experiment using L054 formulation
nanoparticles indicates that these particles were serum stable "such that the
nucleic acid is not substantially degraded after exposure of the particle to a
nuclease at 37ºC for 20 minutes." *See* Mot., Appendix A (proposed
substitute claim 21). As Dr. Thompson testifies "as soon as you feed your
cells, any exposed nucleic acid is shredded. Even in in vitro setting."
Ex. 2019, 129:4–5; *see also id*. at 216–217 (Dr. Thompson stating that "if
you have serum present, if it's not a serum stable formulation, and one that
actually performs in the presence of serum, game over").

We find that proposed substitute claim 21 is unpatentable as
anticipated by the '554 Publication. For the same reasons as set forth above
regarding the anticipation of claims 2–6, 9, 12, 14, and 15, we also find that
substitute claims 22–26, 29, 32, 34, and 35 are also anticipated. Therefore,
we deny Patent Owner's Contingent Motion to Amend.

III.   CONCLUSION

After reviewing the information presented in the Petition and the
Patent Owner Response, as well as the evidence of record, we determine that
Petitioner has shown by a preponderance of the evidence that claims 1–6, 9,
12, 14, and 15 of the '435 patent are unpatentable as anticipated by the '554

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Publication. We also determine that Petitioner has not shown by a preponderance of the evidence that claims 7, 8, 10, 11, 13, or 16–20 are unpatentable.

Because Patent Owner's Motion to Amend was contingent, we only considered the proposed substitute claims for the unpatentable claims, which are substitute claims 21–26, 29, 32, 24, and 35. We found that these proposed substitute claims are unpatentable as anticipated by the '554 Publication.

IV.   ORDER

Accordingly, it is:

ORDERED that claims 1–6, 9, 12, 14, and 15 of U.S. Patent No. 9,364,435 B2 are determined to be unpatentable;

FURTHER ORDERED that claims 7, 8, 10, 11, 13 and 16–20 of U.S. Patent No. 9,364,435 B2 have not been shown by a preponderance of the evidence to be unpatentable

FURTHER ORDERED that Patent Owner's Motion to Amend is *denied*; and

FURTHER ORDERED that, because this is a final written decision, parties to this proceeding seeking judicial review of our Decision must comply with the notice and service requirements of 37 C.F.R. § 90.2.

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JOINT APPENDIX 29

UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE PATENT TRIAL AND APPEAL BOARD

_____

MODERNA THERAPEUTICS, INC.,
Petitioner,

v.

PROTIVA BIOTHERAPEUTICS, INC.,
Patent Owner.

_____

Case IPR2018-00739
Patent No. 9,364,435

_____

DECLARATION OF DAVID H. THOMPSON, PH. D.

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I, David H. Thompson, declare as follows:

I.   QUALIFICATIONS

1.   I am a Professor of Chemistry at Purdue University and Director of the Medicinal Chemistry Group in the Purdue Center for Cancer Research. My primary research interests include development of transiently-stable carrier systems for drug and nucleic acid delivery.

2.   I received my Ph.D. in Organic Chemistry from Colorado State University in 1984. I also hold a Bachelor of the Arts in Biology and a Bachelor of Science in Chemistry from the University of Missouri, Columbia.

3.   I have been a visiting professor at numerous institutions including, Chulalongkorn University, Department of Pharmaceutics; Technical University of Denmark, Department of Micro & Nanotechnology; Japan Advanced Institute of Science & Technology, Department of Biomaterials; Osaka University, Department of Applied Chemistry; University of Florida, Department of Pharmaceutics; and University of British Columbia, Department of Biochemistry.

4.   I am listed as a co-inventor on 7 United States patents. I have also published more than 140 peer reviewed scientific papers.

5.   I have studied, taught, practiced, and conducted research involving the formulation, use, characterization, and delivery of lipid particles. I have expertise with the delivery of therapeutic agents using lipid particles.

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6.   A copy of my Curriculum Vitae, attached as EX2010, contains further details on my education, experience, publications, and other qualifications to render an expert opinion in this matter.

II.   SCOPE OF WORK

7.   I understand that a petition was filed with the United States Patent and Trademark Office for inter partes review of U.S. Patent No. 9,364,435 ("the '435 patent," EX1001).

8.   I further understand that the Patent Trial and Appeal Board ("PTAB" or the "Board") has decided to institute inter partes review of claims 1-20 of the '435 patent under 35 U.S.C. §§ 102 and 103 based on the disclosures of WO2005/007196 ("the '196 PCT," EX1002), US 2006/134189 ("the '189 PCT," EX1003), Lin, et al, "Three-Dimensional Imaging of Lipid Gene-Carriers: Membrane Charge Density Controls Universal Transfection Behavior in Lamellar Cationic Liposome-DNA Complexes," ("Lin," EX1005), Ahmad, et al, "New multivalent cationic lipids reveal bell curve for transfection efficiency versus membrane charge density: lipid–DNA complexes for gene delivery," ("Ahmad," EX1006), and US 2006/0240554 ("the '554 publication," EX1004).

9.   I have been specifically asked to provide my expert opinions on the patentability of the claims of the '435 patent in view of the asserted grounds in the petition. In connection with this analysis, I have reviewed the '435 patent and the

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prior art cited against the patentability of claims 1-20. I have also reviewed and considered the petition, Dr. Janoff's declaration and deposition transcript, and the Board's Decision on Institution of Inter Partes Review, and may cite these documents in this declaration.

10.   I am being compensated at a rate of $600 per hour for my work in this matter. I am also being reimbursed for reasonable and customary expenses associated with my work in this investigation. My compensation is not contingent on the outcome of this matter or the specifics of my testimony.

III.   LEGAL STANDARDS

11.   I have been advised that a claimed invention is not patentable under an anticipation theory (35 U.S.C. § 102) if all claim elements are found in a single prior art reference. I further understand that anticipation is about prior invention and therefore the single prior art reference must be found to disclose all elements of the claimed invention arranged as in the claim. I also understand that picking, choosing, and combining various embodiments disclosed within a single reference is not proper under an anticipation theory.

12.   I understand that differences between the prior art reference and a claimed invention, however slight, invoke the question of obviousness, not anticipation.

13.   I have been advised that a claimed invention is not patentable under 35 U.S.C. § 103 if it is obvious. A patent claim is unpatentable if the claimed invention would have been obvious to a person of ordinary skill in the field at the time the claimed invention was made. This means that even if all of the requirements of the claim cannot be found in a single prior art reference that would anticipate the claim, a person of ordinary skill in the relevant field who knew about all this prior art would have come up with the claimed invention.

14.   I have further been advised that the ultimate conclusion of whether a claim is obvious should be based upon several factual determinations. That is, a determination of obviousness requires inquiries into: (1) the level of ordinary skill in the field; (2) the scope and content of the prior art; (3) what difference, if any, existed between the claimed invention and the prior art; and (4) any objective indicia of nonobviousness.

15.   I have been advised that, in determining the level of ordinary skill in the field that someone would have had at the time the claimed invention was made, I should consider: (1) the levels of education and experience of persons working in the field; (2) the types of problems encountered in the field; and (3) the sophistication of the technology.

16.   I have been advised that a patent claim composed of several elements is not proved obvious merely by demonstrating that each of its elements was

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independently known in the prior art. In evaluating whether such a claim would have been obvious, I may consider whether there is a reason that would have prompted a person of ordinary skill in the field to combine the elements or concepts from the prior art in the same way as in the claimed invention.

17.   I have also been advised, however, that I must be careful not to determine obviousness using the benefit of hindsight; many true inventions might seem obvious after the fact. I should put myself in the position of a person of ordinary skill in the field at the time the claimed invention was made and I should not consider what is known today or what is learned from the teaching of the patent.

18.   Finally, I have been advised that any obviousness rationale for modifying or combining prior art must include a showing that a person of ordinary skill would have had a reasonable expectation of success.

19.   With regard to objective indicia of nonobviousness, I have been advised that any objective evidence may be considered as an indication that the claimed invention would not have been obvious at the time the claimed invention was made. I understand that the purpose of objective indicia is to prevent a hindsight analysis of the obviousness of the claims.

20.   I have been advised that there are several factors that may be considered as objective indicia. These factors include the long-felt need, skepticism, unexpected results and commercial success of the invention.

21.   I have been further advised that in order for objective indicia to be significant, there must be a sufficient nexus between the claimed invention and the evidence of objective indicia. I understand that this nexus serves to provide a link between the merits of the claimed invention and the evidence of objective indicia provided.

IV.   LEVEL OF ORDINARY SKILL IN THE ART

22.   I have been advised that, in determining the level of ordinary skill in the art that someone would have had at the time the claimed invention was made, I should consider: (1) the levels of education and experience of persons working in the field; (2) the types of problems encountered in the field; and (3) the sophistication of the technology. I have been advised that an invention must be evaluated not through the eyes of the inventor, who may have been of exceptional skill, but as by one of ordinary skill in the art.

23.   I understand that Dr. Janoff defined a person of ordinary skill as one that "would have specific experience with lipid particle formation and use in the context of delivering therapeutic payloads, and would have a Ph.D., an M.D., or similar advanced degree in an allied field (*e.g.*, biophysics, microbiology,

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biochemistry) or an equivalent combination of education and experience." EX1007

¶31. I understand this was adopted by the Board. Paper 15 at 5-7.

24.   In my opinion, the level of ordinary skill defined by Dr. Janoff and the

Board is inappropriate. With regard to the "specific experience," Dr. Janoff states

that the "level of skill is representative of the inventors on the '435 patent and

authors/inventors of prior art cited herein." EX1007 ¶31. The inventors of the '435

patent, however, are artisans of exceptional skill in the subject matter of the '435

patent. Thus, in my view, Dr. Janoff has not simply applied a slightly higher level

of skill in the art in setting forth his opinions in his declaration, but has assumed a

much higher level of skill than that of a person of ordinary skill in the art.

V.   BACKGROUND

25.   An objective of genetic therapy at the time, and to this day, is the

development of drugs — that is, nucleic acids — to treat systemic diseases such as

cancer, inflammation, virus infection, and cardiovascular disease. While genetic

therapy holds the promise of highly specific targeting of disease pathways, it was

known that this promise would only be realized through the development of

appropriate delivery vehicles. Delivery is critical because a therapeutic agent is

useless if it does not reach its target. This is particularly true with nucleic acids —

large, negatively charged molecules — that cannot simply be given to a patient

systemically (*e.g.*, intravenously) and allowed to passively enter cells, as would be

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the case with many small molecule drugs. Therapeutic nucleic acids require an effective delivery vehicle, which historically has proved to present a considerable technical obstacle. *See, e.g.*, EX2016 ("You can write down the steps. You can write down what you think will happen. But then you have to put it in a 50-nanometer particle that's safe and potent to deliver."); EX2014 at 11 ("The major hurdle right now is delivery, delivery, delivery," says Sharp), ("Khvorova believes that the medical benefits of RNAi will be huge if the delivery issues can be resolved.").

26.   The first generation of nucleic acid delivery systems that were developed included cationic liposome nucleic acid complexes (also known as liposomes). *See* EX1002 ¶8 (defining "cationic liposome complex" as lipoplex); EX2007, 2:27-28 (same). Lipoplexes were found to be unsuitable for many applications, particularly systemic uses, due in large part to the toxic nature of the cationic lipids. *See, e.g.*, EX1008 at 5.

27.   The toxicity of cationic lipids is observed both at the systemic level and at the cellular level. Cationic lipids cause clustering of membrane glycoproteins on cell surfaces, thereby disrupting normal cellular protein trafficking and receptor recycling, and thus are cytotoxic to cells themselves. Toxicity also occurs at the organ level, as these lipids are often not readily biodegradable, such that they accumulate to cytotoxic concentrations in the liver

and spleen. Cationic lipids also have immunostimulatory capacity and have been associated with immunogenic and inflammatory responses. The presence of cationic lipids also results in these complexes being rapidly cleared from the body, further limiting their therapeutic utility. Furthermore, it was understood that the cationic lipid component can cause aggregation of lipid particles.

28.   These technical obstacles of toxicity, immunogenicity, and aggregation due to use of cationic lipids in a delivery vehicle for nucleic acids was well known in 2008 and thus those in the field at the time sought to minimize the cationic lipid component of a lipid delivery vehicle. This is evidenced in the references cited in the petition.

29.   For example, Ahmad teaches that the cationic lipid component should be minimized to reduce cytotoxicity and metabolic effort associated with elimination of cationic lipids.

> Minimizing the amount of cationic lipid is desirable to reduce cost as well as potential toxic effects of the cationic lipid. In addition, achieving a given σ_M with fewer, more highly charged molecules should mean a *smaller metabolic effort for the elimination of the lipids* from the cell.

EX1006 at 7.

30.   Gao discusses toxicity caused by the cationic lipid component.

> Detailed toxicological studies … revealed that the cationic lipid contributes significantly to the toxicity observed. Similar toxic effects

are also noticeable in systemic gene delivery via the tail vein with other types of cationic lipids. Symptoms include acute pulmonary hypotension, induction of inflammatory cytokines, tissue infiltration of neutrophils in lungs, decrease in white cell counts, and in some cases tissue injury in liver and spleen. In humans, various degrees of adverse inflammatory reactions, including flulike symptoms with fever and airway inflammation, ….

EX1008 at 5.

31.   Gao further discloses that the cationic lipid component caused

unwanted interactions with serum proteins, including complement.

Another factor related to the severity of transfection-related side effects is complement activation and adsorption of serum proteins onto their surface, which in turn act as opsonins to trigger the uptake of opsonized particles by macrophages and other immune cells. Various strategies have been considered to deal with the toxic responses.

EX1008 at 5-6; *see also* Table 1.

32.   Gao also describes that the cationic lipid component of lipoplexes

caused unwanted interactions with non-target cells.

Once administered in vivo, lipoplexes tend to interact with negatively charged blood components and form large aggregates that could be absorbed onto the surface of circulating red blood cells, trapped in a thick mucus layer, or embolized in microvasculatures, preventing them from reaching the intended target cells in the distal location.

EX1008 at 5.

33. Additionally, it was appreciated that the cationic lipid component of lipoplexes caused aggregation of lipid particles. EX1008 at 9 ("[T]he polycations in either lipoplexes or polyplexes have the intrinsic property of causing significant aggregation in biological matrices full of negatively charged molecules …."); *see also* EX1004 ¶136.

34. Therefore, it was well established at the time of filing of the patent that cationic lipids used in a delivery vehicle for nucleic acids were toxic, immunogenic, and caused aggregation. As Dr. Zamore of Alnylam stated, "I wouldn't want anyone injecting cationic lipids into my bloodstream." EX2011 at 42.

35. At the filing date of the patent, the aim of those working in the field sought lipid particles that were substantially non-toxic and therefore suitable for systemic applications. However, at that time, the development of nucleic acid-lipid particles that were suitable for systemic applications had not been achieved. Furthermore, it was widely understood at that time that in order to design nucleic acid-lipid particles suitable for systemic use the amount of cationic lipid in the formulation should be kept as low as possible, because of concerns over the known toxic effects of cationic lipids.

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VI.   CLAIM CONSTRUCTION

36.   The petition materials provided an unreasonably broad construction of "nucleic acid-lipid particle," stating that it should be construed as "a composition of lipids and a nucleic acid for delivering a nucleic acid to a target site on interest." Pet. 24.

37.   I have been apprised that the Board, in its Institution Decision, rejected the construction in the petition and offered a different one. That is, the Board construed "nucleic acid-lipid particle" as "a particle that comprises a nucleic acid and lipids, in which the nucleic acid may be encapsulated in the lipid portion of the particle." Paper 15 at 10-11 (citing EX1001, 11:14–22).

38.   In my opinion, both constructions of "nucleic acid-lipid particle" in the petition materials and in the Institution Decision are incorrect and unreasonably broad at least to the extent they encompass lipid particles lacking any encapsulated nucleic acid. The petition materials and the Board focused on a different term — *i.e.*, the term "lipid particle" — and only incompletely address the corresponding discussion in the '435 patent specification. But the claimed term is not "lipid particle," the claimed term is "nucleic acid-lipid particle."

39.   A "nucleic acid-lipid particle" expressly includes a nucleic acid. According to the '435 patent, "nucleic acids, when present in the lipid particles of the present invention, are resistant in aqueous solution to degradation with a

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nuclease." EX1001, 11:51-54. The '435 patent describes nucleic acid encapsulation in the lipid particle as conferring resistance to such enzymatic degradation. EX1001, 11:20-22 ("[T]he active agent or therapeutic agent may be encapsulated in the lipid, thereby protecting the agent from enzymatic degradation."); *see also* EX2007 4:15-19; 22:40-47; 23:1-3; 23:27-29; 26:35-37 (describing resistance to nuclease enzymatic degradation as indicating nucleic encapsulation in the liposomes).

40.   A "lipid particle" "*may* [include a nucleic acid] encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation." EX1001, 11:14–22. A "nucleic acid-lipid particle," however, *does* include a nucleic acid encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation. EX1001, 11:23-31, 11:51-54.

41.   I understand that, during cross-examination, Dr. Janoff testified multiple times that the lipid particles as claimed are defined as SNALPs. *See, e.g.*, EX2028, 118:18-119:4, 119:9-17, 120:5-6, 121:14-25. Dr. Janoff cited to a provision of U.S. Patent No. 9,404,127 ("the '127 patent," EX2029) at 5:15-22 that is identically recited in the '435 patent. *Compare* EX2029, 5:15-22 *with* EX1001, 19:19-26.

42.   Dr. Janoff is correct in that the specification repeatedly identifies SNALPs as the invention of the patent for delivering a nucleic acid payload. *See*

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e.g., EX1001, 3:9-13 ("The present invention provides novel, serum-stable lipid

particles …."), 47:23-24 ("[T]he lipid particles of the invention are serum-stable

nucleic acid-lipid particles (SNALP)…"), 3:32-37, 14:20-25.

43.   In my opinion, a fair and reasonable reading of the '435 patent

specification supports Dr. Janoff's position in that there is no meaningful

distinction between the '435 patent specification's descriptions of a "lipid particle"

containing a nucleic acid (*i.e.*, a nucleic acid-lipid particle) and particle

characteristics that confer serum stability. *Compare* EX1001, 11:14-22, 11:51-54

("[N]ucleic acids, when present in the lipid particles of the invention, are resistant

in aqueous solution to degradation with a nuclease.") *with* 13:32-37 ("'Serum-

stable' in relation to nucleic acid-lipid particles such as SNALP means that the

particle is not significantly degraded after exposure to a serum or nuclease assay

that would significantly degrade free DNA or RNA").

44.   In my opinion, a narrow focus on a linguistic difference between a

nucleic acid-lipid particle and the term "SNALP" is misguided and risks

overlooking pertinent disclosure and context provided in the '435 patent. The '435

patent specification states that "nucleic acids, when present in the lipid particles of

the invention, are resistant in aqueous solution to degradation with a nuclease." *Id.*,

11:51-54. Such physical properties of the particles providing nuclease degradation

resistance, or encapsulation of the nucleic acid, are also as described in the '435

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patent specification as conferring the identified serum stability. *Id.*, 13:32-37. This

is certainly true if the claimed particles are SNALP, as supported not only by the

specification of the '435 but as affirmed by petitioner's expert Dr. Janoff.

45.   I disagree with the Board's analysis presented at pages 9-10 of the

Institution Decision at least for the reasons explained above. As explained above,

there is no meaningful distinction between a nucleic acid-lipid particle and a

SNALP in the context of the '435 patent. None of the provisions of the '435 patent

specification identified by the Board indicate otherwise. Paper 15 at 9-10. The

Board's discussion of whether particles are "limited to in vivo use," is confusing

and loses sight of both the context of the '435 patent specification and a reasonable

perspective of a person of ordinary skill in the art. For example, the Board cites to

an example of SNALP being tested for transfection activity in vitro as indicating

the same composition is not a SNALP. Paper 15 at 10 (citing Example 2 at 69:6–

70:52 ("Eg5 siRNA Formulated as 1:57 SNALP are Potent Inhibitors of Cell

Growth In Vitro")). The composition in Example 2 is expressly described as "1:57

SNALP." A person of ordinary skill in the art would understand that composition

can be both 1) formulated such that the nucleic acid is encapsulated in the lipid

particle, rendering the composition extremely useful for systemic applications; and

2) tested for in vitro transfection activity. It is not uncommon for compositions to

be assessed for in vitro transfection activity and then subject to testing in vivo.

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46.   Regardless of whether the Board construes "nucleic acid-lipid particle" as a SNALP as indicated by Dr. Janoff; as a lipid particle with an encapsulated nucleic acid (thereby protecting it from enzymatic degradation); or under the broad construction presented in the Institution Decision, the petition materials fail to establish the unpatentability of claims 1-20.

VII.   GROUND 1 – CLAIMS 1-20 ARE NOT OBVIOUS IN VIEW OF PATENT OWNER'S DISCLOSURES IN THE '196 PCT AND '189 PUBLICATION

47.   It is my opinion that the petition fails to demonstrate that claims 1-20 of the '435 patent are obvious in view of the '196 PCT and the '189 publication. Since the petition materials provide no meaningful discussion of the '189 publication, the below arguments focus on lack of obviousness in view of the '196 PCT.

48.   Although I understand that it is not Patent Owner's burden to prove, it is my opinion that claims 1-20 of the '435 patent are not obvious.

A.   Claim 1

49.   First, the petition materials fail to address all the lipid components of the claimed nucleic acid-lipid particle composition. Second, the petition materials fail to address the combination of the lipid components of the claimed nucleic acid-lipid particle composition. Third, the petition materials fail to explain why a person of ordinary skill in the art would have wanted to combine the individual lipid

components disclosed in the '196 PCT. Fourth, the petition materials fail to

address that a skilled artisan would have had no reasonable expectation of success

that the claimed nucleic acid-lipid particle composition would be well-tolerated

and efficacious. Finally, much of the evidence of unexpected results within the

patent is disregarded, and what is considered is mischaracterized in view of the

prior art.

50.   As an initial observation, the assertion of obviousness in the petition

materials is based on alleged overlapping ranges between the '196 PCT and the

challenged claims. But the petition materials fail to identify ranges that overlap for

each of the claim components.

51.   For example, claim 1 recites "a conjugated lipid that inhibits

aggregation of particles." Rather than identifying disclosure in the '196 PCT that

is specific for a conjugated lipid range, the petition materials cite to a range

provided for "a bilayer stabilizing component." Pet. 39; EX1007 ¶117.

52.   The '196 PCT makes clear that a "bilayer stabilizing component" is

not the same as a "conjugated lipid that inhibits aggregation of particles." *See, e.g.*,

EX1002 ¶92 ("Suitable BSCs include, but are not limited to, polyamide oligomers,

peptides, proteins, detergents, lipid-derivatives, PEG-lipids, …"). Bilayer

stabilizing components include a broad class of structurally and chemically diverse

molecules. Numerous bilayer stabilizing components (*e.g.*, polyamide oligomers,

peptides, proteins, detergents, and lipid-derivatives) would not be considered a conjugated lipid by a person of ordinary skill in the art.

53.   While the '196 PCT lists a general range for the bilayer stabilizing component category, a person of ordinary skill in the art would not have interpreted the stated range (*e.g.*, 0.5% to 25%) as being applicable to each listed bilayer stabilizing component example. For example, a skilled artisan would have appreciated that if the bilayer stabilizing component were a detergent, 25% would have been an unreasonably high level. This is because at this concentration of detergent the lipids would be solubilized and no longer form a lipid particle.

54.   The '196 PCT discloses seven nucleic acid-lipid particle compositions — all of which have either 7.5 or 15 mol % cationic lipid and 10 mol % conjugated lipid. EX1002 ¶¶216, 223, 228, 232.

55.   Therefore, the petition materials fail to identify in '196 PCT "a conjugated lipid that inhibits aggregation of particles" as required by claim 1.

56.   In my opinion, as explained in further detail below, there would have been no good reason why a person of ordinary skill in the art would combine the different range disclosures for different lipid components from the '196 PCT so as to arrive at the claimed nucleic acid-lipid particle. Nor would one would reasonably expect such formulations to work. The petition materials fail to demonstrate otherwise.

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57.   Those in the field at the time recognized that the properties of any

lipid particle are conferred not by the amount of any individual component but by

the interaction of the combined components as a whole. Furthermore, if the skilled

person were to vary one component (by taking the amount of that component

specified for a particular formulation), it would then be necessary to decide which

of the other components would need to be varied in order to accommodate the

change in proportions of the overall composition.

58.   The effects of making changes to the proportion of other components

in the lipid particle would be unpredictable. Such changes, even if apparently

minor in nature, would have little assurance of producing a functional lipid particle

suitable for systemic use. The idea of simply "cherry-picking" specific amounts of

individual components from different formulations, or the different ranges in the

'196 PCT, when designing lipid particles is therefore something which would have

made no technical sense to the skilled person.

59.   Dr. Janoff states that "determining the optimal proportion of cationic

lipid for a given lipid combination would be a simple matter of varying the

proportion using prior art methodologies." EX1007 ¶110. I disagree. As explained

above, the properties of a formulation are not conferred by the amount of one

single component. Properties such as safety and efficacy are conferred by the

combination of components in the entire formulation.

60.   Moreover, Dr. Janoff's stated reason disregards the state of the art at the time of the invention. Making safe and effective nucleic acid-lipid particle formulations was not simply a matter of "varying the proportion" of cationic lipid in prior art formulations. As discussed above, the field of genetic medicine was hindered by the lack of effective and safe nucleic acid delivery vehicles. That the field struggled for 20 years to find such a delivery vehicle speaks to the difficulty of the task. *See generally* EX2015. Had the solution been a matter of simply optimizing the cationic lipid proportion, it would not have taken such an enormous investment of money and time.

61.   As discussed elsewhere herein, the high cationic lipid levels claimed would have been disfavored in view of well-established toxicity concerns. Moreover, inclusion of a conjugated lipid in a formulation with high cationic lipid would have been expected to occur at much higher levels than claimed.

62.   Conjugated lipid had been incorporated into lipid particles to help shield positive charge and reduce nonspecific interactions with blood components, leading to enhanced systemic clearance. Lipid particle compositions at the time typically used much higher levels of conjugated lipid than is claimed by the '435 patent, such as 10% PEG (*i.e.*, 5- to 20-times more than the claimed formulations). For example, Doxil, the first FDA approved liposome formulation contained 5% PEG-conjugated lipid. EX2034. Likewise lipid particles for the delivery of nucleic

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acids commonly used 10% PEG. EX2032 at 174; EX2033 at 1021; EX1002 ¶¶216, 223, 228, 232.

63.   The petition materials fail to provide a reasonable expectation of success for the claimed nucleic acid-lipid particle in view of the '196 PCT and the state of the art at the date of the patent filing. In my opinion, a person of ordinary skill in the art would not have expected a nucleic acid-lipid particle composition with a high level of cationic lipid and a low level of conjugated lipid to be safe and effective.

64.   As discussed above, the prior art taught that the cationic lipid component of lipid particles should be minimized, regardless of whether used for in vitro or in vivo purposes. For example, it was appreciated that cationic lipids are directly cytotoxic. Cationic lipids, in addition, elicit unwanted immune reactions (*e.g.*, inflammation), off-target cellular interactions (*e.g.*, blood cells), and aggregation. Furthermore compositions with low levels of conjugated lipid (*i.e.*, 0.5 mol % to 2 mol %) would have been expected to result in unstable particles that aggregate and fail to effectively transfect cells. Hence the claimed nucleic acid-lipid particle would have been expected to be cytotoxic and ineffective.

65.   Accordingly, claim 1 is not obvious in view of the '196 PCT at least because there would have been no expectation of success that the claimed nucleic acid-lipid particle would be safe and effective.

B.   Unexpected Results

66.   I reviewed the experimental data in the '435 patent. In my opinion,

the results of the experimental data would have been quite surprising to a POSITA.

As explained in further detail below, those in the art at the time would have

expected formulations as claimed to result in significantly lower activity and

higher levels of toxicity compared to what is reported in the experimental results.

67.   The prior art expressly instructs that high-level cationic lipid

formulations were expected to have poor *in vivo* activity and display increased

toxicity and immunogenicity relative to low-level cationic lipid formulations. *See*

EX1005 at 3315; EX1006 at 745; EX1008 at E96; EX2007, 30:34:41 (Issued

patent naming Dr. Janoff as an inventor and explaining that "[t]here may be a limit

to the use of cationic lipoplexes [in vivo] because of their toxicity."). As such, the

expectation for the claimed formulations would have been toxic formulations

unsuitable for systemic use and little, if any, efficacy.

68.   Contrary to these expectations, the claimed formulations are well-

tolerated and possess favorable *in vivo* efficacy at far lower dosages than prior art

formulations. Data for numerous formulations within the scope of claim 1 are

found in the '435 patent and support the unexpected degree of tolerability and

efficacy. *See also* Section XI.

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69.   The petition materials present an incomplete and flawed analysis of

the data within the '435 patent. First, the petition materials fail to consider that,

unexpectedly, the claimed formulations are not toxic nor do they cause unwanted

immune responses. Second, although the petition materials purport to address the

data supporting unexpected efficacy, these data are analyzed without consideration

of what a person of ordinary skill in the art would have expected.

70.   Dr. Janoff says "the *sole basis* for alleged novelty of the '435 patent

claims is that a nucleic acid-lipid particle comprising component lipids in the

claimed proportions achieves *unexpected efficacy* making the claims patentably

distinct from the prior art." EX1007 ¶75 (emphases added). Not so. The '435

patent also discloses that the claimed formulations "are substantially non-toxic to

mammals such as humans." EX1001, 6:2-5; *see also* EX1001, 6:26-30, 11:51,

14:40-42, 23:2-3, 47:9-18.

71.   For example, the '435 patent used various measurements (*e.g.*, body

weight, appearance and behavior, and platelet count) to assess the toxicity of the

claimed compositions following systemic administration to mice. Unexpectedly,

no significant toxicity was detected by any measure.

72.   Body weight was monitored after systemic administration of a 1:57

formulation. EX1001, 79:33-37. A sharp decline was considered indicative of

toxicity and would have been the expected result. However, "there was very little

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effect on body weight 24 hours" even at the highest dosages. EX1001, 79:33-34; *see also id.*, 79:34-36 ("The maximum weight loss of 3.6±0.7% was observed at the highest drug dose of 17 mg/kg."), Figure 8. Moreover, the '435 patent explains that "[t]here was also no obvious change in animal appearance/behavior at any of the dosages tested." EX1001, 79:36-37.

73.   Platelet count was also measured after systemic administration of a 1:57 nucleic acid-lipid particle composition. An increase or decrease in platelet count is indicative of toxicity and would have been the expected result. *See* EX1001, 79:38-41. However, "the mean platelet volume did not change in SNALP-treated groups." EX1001, 79:44; *see also* Figure 9.

74.   No significant toxicity was observed even after multiple systemic doses of the 1:57 nucleic acid-lipid particle composition. Mice were dosed intravenously multiple times per week for a total of 5 weeks. EX1001, 81:20-82:25. Similar to the single dose of 1:57, no treatment-related toxicity was observed. EX1001, 82:30-36 ("The treatment regimen was well tolerated with no apparent signs of treatment-related toxicity."), ("[T]reatment with 1:57 SNALP-formulated PLK1424 caused a significant increase in the survival of Hep3B tumor-bearing mice. This in vivo anti-tumor effect was observed in the absence of any apparent toxicity or immune stimulation.").

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75.   For example, the claimed nucleic acid-lipid particle compositions effectively induced silencing of the targeted gene (*i.e.*, ApoB) when administered systemically to mice. Table 4 lists the nucleic acid-lipid particle compositions tested as part of Example 3.

TABLE 4

			Finished Product Characterization		
Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Size (nm)	Poly-dispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2 | 40 | 10 | 48	12.4	59	0.15	93
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 2.2 | 44.4 | 53.3	10.7	55	0.17	91
4	PEG(2000)-C-DMA | DLinDMA | DOPC | Cholesterol 2 | 40 | 10 | 48	12.5	59	0.16	92
5	PEG(2000)-C-DMA | DLinDMA | DMPC | Cholesterol 2 | 40 | 10 | 48	12.2	56	0.11	92
6	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.8 | 36.4 | 18.2 | 43.6	13.8	66	0.16	93
7	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 2 | 40 | 10 | 48	12.4	56	0.12	92
8	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 27.0 | 6.8 | 64.9	16.5	60	0.10	93
9	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.3 | 25.3 | 12.7 | 60.8	18.1	74	0.13	92
10	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2.5 | 25.0 | 12.5 | 60.0	19.2	60	0.13	93
11	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.4 | 34.3	17.8	79	0.09	94
12	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.0 | 40.4 | 10.1 | 48.5	23.6	72	0.11	93
13	PEG(2000)-C-DMA | DLinDMA | DPPC 2 | 70 | 28	8.7	73	0.09	87
14	PEG(2000)-C-DMA | DLinDMA | DPPC 1.6 | 54.7 | 43.8	11.3	65	0.11	87

Characteristics of the SNALP formulations used in this study.

EX1001, Table 4. Amongst those tested were nucleic acid-lipid particle compositions within the scope (*i.e.*, Groups 11, 13, and 14) and outside the scope of claim 1. The expectation for nucleic acid-lipid particle compositions of Groups 11, 13, and 14 would have been little, if any, gene silencing. That is, based on what was known in the art at the time, Groups 11, 13, and 14 would have been expected

to yield reductions in gene expression similar to the "untreated" control group (*i.e.*, PBS). However, each of the nucleic acid-lipid particle compositions within the scope of claim 1 (Groups 11, 13, and 14) showed significant gene silencing relative to the control group (*i.e.*, PBS). EX1001, FIG 2. Even more surprisingly, these nucleic acid-lipid particle compositions yielded gene silencing levels which were at least comparable and in many cases superior to prior art compositions that have a much lower cationic lipid level (*e.g.*, 25 mol % to 40 mol % cationic lipid). EX1001, FIG 2 (red and green highlighting added).
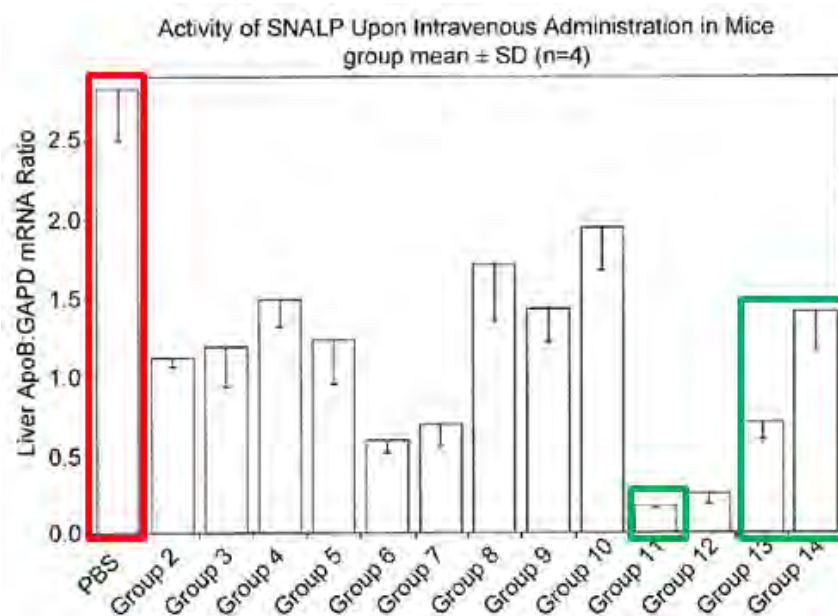
For instance, the 1:57 nucleic acid-lipid particle composition, "was substantially more effective at silencing the expression of a target gene as compared to prior art nucleic acid-lipid particles ('2:40 SNALP')." EX1001, 6:10-14. Furthermore, the

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1:57 nucleic acid-lipid particle composition "was the most potent at reducing" gene expression in vivo. EX1001, 72:25-27.

76.   The 1:57 nucleic acid-lipid particle composition was also compared to the prior art 2:30 composition. Surprisingly, the 1:57 nucleic acid-lipid particle composition was "more than 10 times as efficacious as the 2:30 SNALP" composition in silencing gene expression in mouse liver when administered systemically. EX1001, Figure 3 (reproduced below). Remarkably, the 1:57 nucleic acid-lipid particle composition achieved these results at "a 10-fold lower dose" than the 2:30 composition. That is, even at a 10-fold lower dose, the claimed nucleic acid-lipid particle composition produced more gene silencing *in vivo* than the prior art composition.
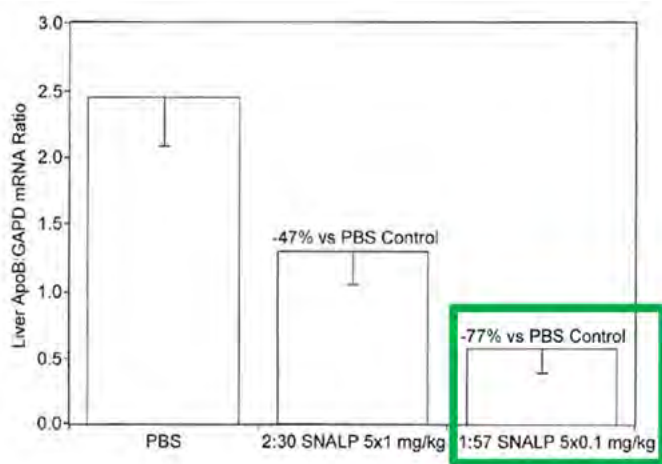
EX1001, FIG 3 (green highlighting added).

77.   Dr. Janoff states that "[a]t most, [Example 4] established that the 1:57 SNALP comprised of the specific species of lipid components and nucleic acid to

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lipid ratio disclosed, dosed as disclosed, outperformed the 2:30 SNALP comprised

of the lipid species disclosed and dosed as disclosed." EX1007 ¶85. Dr. Janoff

further states that "given the disclosures in the '435 patent, a POSITA would not

expect all alternative data points falling within the recited numeric range to

perform like the 1:57 SNALP." EX1007 ¶112. These comments disregard the

breadth of formulations tested. As discussed below, Examples 5 and 6 describe

testing of additional formulations within the scope of claim 1 which also yield

considerable levels of gene silencing in vivo. EX1001, 74:5-58. I discuss those

results in further detail below.

78.   Example 5 discloses the testing of seven formulations within the

scope of claim 1. EX1001, 74:11-58, Table 6.

TABLE 6

Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/ Drug Ratio	Finished Product Characterization		
			Size (nm)	Poly-dispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	76	0.06	89
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 1.5 | 61.5 | 36.9	8.1	76	0.04	86
4	PEG(2000)-C-DMA | DODMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.0	72	0.05	95
5	PEG(5000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.6	52	0.16	89
6	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 1.4 | 57.1 | 7.1 | 34.3	8.9	68	0.10	94
7	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	72	0.07	95
8	PEG(2000)-C-DMA | DLinDMA | DPPC 1.8 | 70.2 | 28.1	8.6	74	0.13	86

EX1001, Table 6. Figure 4 displays the level of gene silencing following systemic administration of each of the seven formulations as compared to the performance of a control (*i.e.*, PBS). A person of ordinary skill in the art would have expected these formulations to be ineffective — that is, produce levels of gene silencing similar to that of the control. EX1001, FIG. 4. Contrary to expectations, these formulations produce significant reductions in gene expression.

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
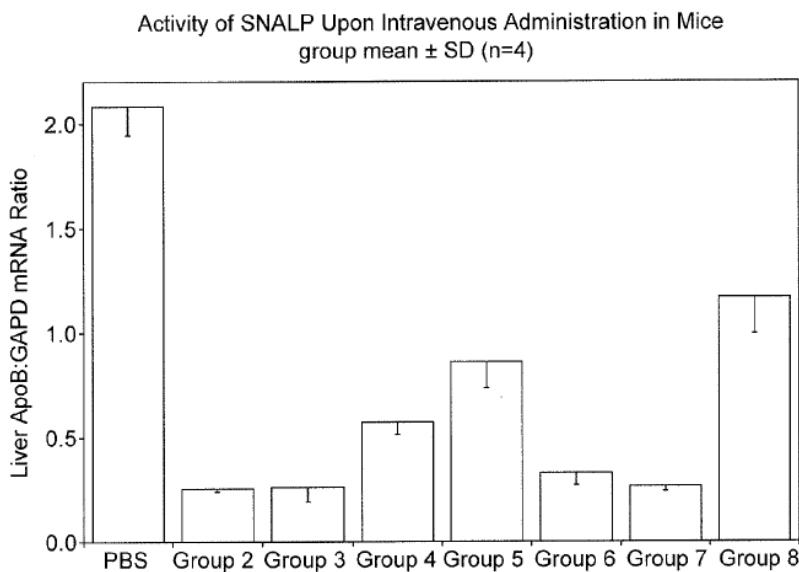
EX1001, FIG 4.

79.   Dr. Janoff states "Example 5 in the '435 patent shows variation of the cationic lipid apparently impacts efficacy" and concludes that "[a] POSITA would understand these results to suggest that a preferred proportion for one cationic lipid (*e.g.*, DLinDMA) does not necessarily apply to all other cationic lipids (*e.g.*, DODMA)." While there is some variation in the efficiency of formulations depending on which cationic lipid is used, (*compare* Group 4 (DODMA) *with*

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Group 2 (DLinDMA)), this does not take away from the conclusion that both DODMA and DLinDMA are unexpectedly efficacious.

80.   Example 6 discloses the testing of 14 formulations within the scope of claim1 (*i.e.*, 54 mol % to 68 mol % cationic lipid). EX1001, 74:11-58, Table 7.

TABLE 7

Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition, Mole % PEG(2000)-C-DMA | DLinDMA | Cholesterol	Lipid/ Drug Ratio	Finished Product Characterization Size (nm)	Poly-dispersity	% Encapsulation
2	1.5 | 61.5 | 36.9	6.1	80	0.07	92
3	1.4 | 54.8 | 43.8	6.6	74	0.05	89
4	2.0 | 61.2 | 36.7	6.2	71	0.11	91
5	1.8 | 54.5 | 43.6	6.7	67	0.09	91
6	1.3 | 68.1 | 30.6	7.4	91	0.06	89
7	1.2 | 61.8 | 37.1	8.0	87	0.10	90
8	1.7 | 67.8 | 30.5	7.6	81	0.07	91
9	1.4 | 56.3 | 42.3	8.6	75	0.11	92
10	1.9 | 61.3 | 36.8	8.2	72	0.10	91
11	1.8 | 56.1 | 42.1	8.8	70	0.10	90
12	1.3 | 66.7 | 32.0	9.5	89	0.09	89
13	1.2 | 61.7 | 37.0	10.0	87	0.10	91
14	1.7 | 66.4 | 31.9	9.6	82	0.11	90
15	1.5 | 61.5 | 36.9	10.1	79	0.10	91

EX1001, Table 7. Figure 5 depicts the level of gene silencing activity achieved with the 14 formulations and compares this activity to the expected result — the performance of the PBS control.

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Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
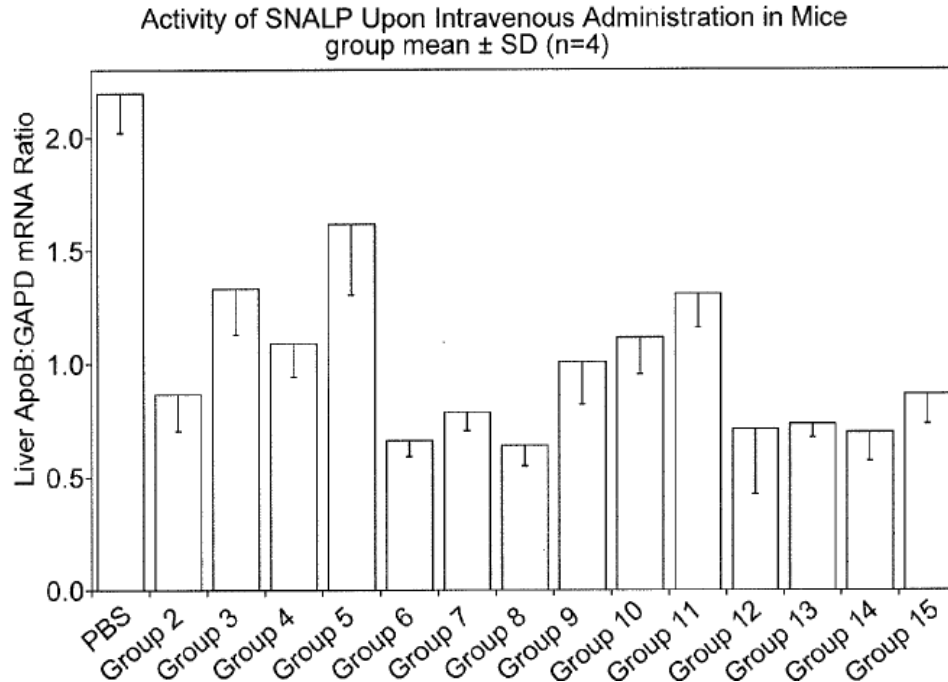
EX1001, FIG 5. Surprisingly, each of the nucleic acid-lipid particle compositions demonstrated significant levels of gene silencing. The '435 patent notes "that the 1:62 SNALP formulation was one of the most potent inhibitors of ApoB expression at two different lipid:drug ratios (*i.e.*, 6.1 & 10.1) among the phospholipid-free SNALP formulations tested (see Groups 2 & 15)." EX1001, 75:45-48.

81.   In sum, the '435 patent discloses data demonstrating nucleic acid-lipid particle compositions across the claimed range are unexpectedly well-tolerated and efficacious.

C.   Claims 2-20

82.   As an initial matter, the analysis of the dependent claims in the petition and Dr. Janoff's declaration is often difficult to follow. For instance,

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Ground 1 alleges obviousness of claims 1-20, but many of the dependent claims include language that appears to invoke an unspecified anticipation theory. *See, e.g.*, EX1007 ¶124 ("Given the breadth of the claimed range for the phospholipid, these disclosures are sufficiently specific to disclose the claimed range. In addition, given the explicit disclosure of encompassing ranges, this limitation is *prima facie* obvious."). Additionally, most of the claims simply map disclosure without any explanation as to its significance. *See, e.g.*, EX1007 ¶124. Finally, the dependency of claims is largely ignored therefore it is challenging to determine what theory of unpatentability is actually being advanced. *See, e.g.*, EX1007 ¶125 (discussing claim 8 which depends from claims 1 and 5). I have done my best to respond to what seems to be the theory of unpatentability for each of the dependent claims.

83.   It is my opinion that claims 2-20 are not obvious for the same reasons identified above with respect to claim 1. Additional discussion for some of claims 2-20 is provided below.

> **Claim 4. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from 50 mol % to 65 mol % of the total lipid present in the particle.**

84.   Dr. Janoff merely refers to his discussion of the cationic lipid level recited and claim 1 and concludes:

> Given the breadth of the claimed range, the disclosures above are sufficiently specific to disclose the claimed range. Not only does the disclosed broader range substantially overlap with the claimed range,

a preferred embodiment in the reference recites a narrower range that
also partially overlaps. In addition, given the explicit disclosure of
overlapping ranges, this limitation is *prima facie* obvious.

EX1007 ¶121. I do not know what range Dr. Janoff asserts belongs to a "preferred

embodiment." Moreover, claim 4 recites a narrower range of cationic lipid than

claim 1 — a range that is well represented by the tested range of cationic lipid

and, in particular, a nucleic acid-lipid particle composition with 57 mol % cationic

lipid was found unexpectedly well-tolerated and efficacious. *See* Section VIII.B.

Thus, the nexus of the unexpected results and claim 4 is even stronger than it is for

claim 1.

> **Claim 5. The nucleic acid-lipid particle of claim 1, wherein the
> non-cationic lipid comprises a mixture of a phospholipid and
> cholesterol or a derivative thereof.**

85.   Neither the petition nor Dr. Janoff points to disclosure of nucleic acid-

lipid particles with a mixture of phospholipid and cholesterol. Instead, reliance is

placed on long lists of non-cationic lipids including phospholipids, sterols, and

non-phosphorous containing lipids and generic ranges for non-cationic lipid and

cholesterol. EX1007 ¶122 (citing EX1002 ¶¶89, 91). These paragraphs do not

disclose nucleic acid-lipid particle compositions that contain a mixture of

phospholipid and cholesterol. To the extent it is argued that a skilled artisan might

select both lipid components for inclusion in a nucleic acid-lipid particle, I

understand that an obviousness theory requires a reason to make such a

combination and a reasonable expectation of success — both of which are absent.

86.   Moreover, claim 5 requires a mixture of phospholipid and cholesterol

such as that found in 1:57 nucleic acid-lipid particle compositions which are

unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of

the unexpected results and claim 5 is even stronger than it is for claim 1.

> **Claim 6. The nucleic acid-lipid particle of claim 5, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.**

87.   Dr. Janoff relies on a long list of non-cationic lipids for disclosure of

DPPC and DSPC. EX1007 ¶123 (citing EX1002 ¶¶89, 128). It is unclear how the

cited disclosure relates to the disclosure cited in the context of claims 1 and 5, from

which claim 6 depends. To the extent that Dr. Janoff is arguing that a skilled

artisan might select DPPC or DSPC from a long list of non-cationic lipids and

incorporate one or both into a nucleic acid-lipid particle composition that includes

cholesterol, I understand that such a theory requires a reason to make the

formulation and a reasonable expectation of success — both of which are absent.

> **Claim 7. The nucleic acid-lipid particle of claim 5, wherein the phospholipid comprises from 3 mol % to 15 mol % of the total lipid present in the particle.**

88.   As an initial matter, the '196 PCT does not disclose a range for a

phospholipid component, much less a range for a phospholipid component in a

nucleic acid-lipid particle composition that includes cholesterol. Instead, Dr. Janoff

arrives at the limitation through arithmetical manipulations of ranges of non-

cationic lipid and cholesterol two different patent documents. EX1007 ¶124.

Specifically, Dr. Janoff selects the 20% to 85% range of non-cationic lipid from

the '196 PCT and subtracts from this range the 20% to 45 % range of cholesterol

disclosed in the '618 patent. A person of ordinary skill in the art would not

consider these calculations to amount to disclosure of a range of phospholipid.

There is no basis to conclude that the '196 PCT provides an "explicit disclosure"

of a range of 0% to 20% phospholipid. EX1007 ¶124. Moreover, the 1:57 nucleic

acid-lipid particle composition is encompassed by claim 7 and is unexpectedly

well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the

unexpected results and claim 7 is even stronger than it is for claim 1.

> **Claim 8. The nucleic acid-lipid particle of claim 5, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle.**

89.   As an initial matter, the '196 PCT does not disclose a range for

cholesterol in a formulation that includes phospholipid. Dr. Janoff relies on

disclosure in the '196 PCT of a range of 20 mol % to 45 mol % cholesterol.

However, this is insufficient to make claim 8, which depends from claims 1 and 5,

obvious. Dr. Janoff also relies on a lipoplex composition in the '618 patent that

contains 30% cholesterol. A person of ordinary skill in the art would not look to

lipoplex compositions for guidance in making nucleic acid-lipid particle

compositions. Moreover, the 1:57 nucleic acid-lipid particle composition is

encompassed by claim 8 and is unexpectedly well-tolerated and efficacious. *See*

Section VIII.B. Thus, the nexus of the unexpected results and claim 8 is even

stronger than it is for claim 1.

> **Claim 12. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises from 1 mol % to 2 mol % of the total lipid present in the particle.**

90.   Neither the petition nor Dr. Janoff provides any reason to use the

range of conjugated lipid of claim 12 in a nucleic acid-lipid particle composition

with the claimed ranges of cationic and non-cationic lipid components. *See*

EX1007 ¶¶119, 120. Any discussion of reasonable expectation of success is

similarly missing. Moreover, claim 12 recites a narrower range of cationic lipid

than claim 1 — a range that is well represented by the tested range of cationic lipid

and, in particular, nucleic acid-lipid particle compositions with 57 mol % cationic

lipid are unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the

nexus of the unexpected results and claim 12 is even stronger than it is for claim 1.

> **Claim 13. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.**

91.   The petition and Dr. Janoff rely solely on one paragraph of the '196

PCT, which states "[i]n some embodiments, the siRNA molecule is fully

encapsulated within the lipid bilayer of the nucleic acid-lipid particle such that the

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nucleic acid in the nucleic acid-lipid particle is resistant in aqueous solution to

degradation by a nuclease." EX1002 ¶11. Dr. Janoff provides no explanation for

why a person of ordinary skill in the art would have had a reasonable expectation

of success generating fully encapsulated nucleic acid-lipid particles that have a

high level of cationic lipid and a low level of conjugated lipid. Among other issues,

a person of ordinary skill in the art would have expected the claimed nucleic acid-

lipid particle to be unstable and thus incapable of encapsulating nucleic acids.

> **Claim 14. A pharmaceutical composition comprising a nucleic
> acid-lipid particle of claim 1 and a pharmaceutically acceptable
> carrier.**

92.   Dr. Janoff provides no explanation for why a person of ordinary skill

in the art would have had a reasonable expectation of success generating a

pharmaceutical composition of nucleic acid-lipid particles that have a high level of

cationic lipid and a low level of conjugated lipid. As discussed above, a person of

ordinary skill in the art would have expected the claimed particles to be prone to

aggregation and to be unstable due to the combination of a high level of cationic

lipid and a low level of conjugated lipid. Consequently, a person of ordinary skill

in the art would not have expected to successfully generate a pharmaceutical

composition.

> **Claim 16. A method for the in vivo delivery of a nucleic acid, the
> method comprising: administering to a mammalian subject a
> nucleic acid-lipid particle of claim 1.**

> **Claim 17. A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising: administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 1.**
>
> **Claim 18. The method of claim 17, wherein the disease or disorder is a viral infection.**
>
> **Claim 19. The method of claim 17, wherein the disease or disorder is a liver disease or disorder.**
>
> **Claim 20. The method of claim 17, wherein the disease or disorder is cancer.**

93.   Dr. Janoff does not include any discussion of reasonable expectation of success. However, the '196 PCT teaches that nucleic acid-lipid particles for systemic use should have cationic lipid in the range of 5 mol % to 15 mol % — that is, much lower than the claimed range. As I discuss in detail above, a skilled artisan would have expected the claimed nucleic acid-lipid particles to be too toxic for in vivo administration. Thus, a person of ordinary skill in the art would not have had a reasonable expectation of success of obtaining nucleic acid-lipid particles suitable for administration to mammalian subjects.

VIII. GROUND 2 – CLAIMS 1-20 ARE NOT OBVIOUS IN VIEW OF PATENT OWNER'S PRIOR DISCLOSURES IN LIGHT OF LIN AND/OR AHMAD

94.   The petition alleges that Lin and Ahmad provide additional support, over "patent owner's prior disclosures." Although Ground 2 alleges that claims 1-20 are obvious, the petition materials only address the cationic lipid limitations of claims 1 and 4. Ground 2 lacks a plausible explanation as to why a person of

ordinary skill in the art would want to combine disclosure regarding "lipoplexes"

with nucleic acid-lipid particle technology as described in the '196 PCT.

Moreover, the statement in the petition materials that a skilled artisan would have

had a reasonable expectation of success is conclusory, nonsensical, and appears to

be based on a misapprehension of the prior art.

95.   The petition materials state that "the Lin and Ahmad systems tested

helper lipids and cationic lipids to create carrier particles for nucleic acids, *i.e.*,

'nucleic acid-lipid particles,' the same general carrier particles described in the

Patent Owner's prior disclosures." Pet. 50. Lin and Ahmad are directed to

lipoplexes. EX1002 ¶8 (defining "cationic liposome complex" as lipoplex);

EX2007, 2:27-28 (same). Lipoplexes are *not* nucleic acid-lipid particles.

96.   Lipoplexes are distinct from the claimed nucleic acid-lipid particles.

Lipoplexes and nucleic acid-lipid particles have different lipid compositions. *See,*

e.g., EX1002, ¶85 (explaining that nucleic acid-lipid particles "comprise a nucleic

acid …, a cationic lipid, a noncationic lipid and a bilayer stabilizing component");

EX1005 at 2 (describing lipoplexes that comprise DNA and cationic and neutral

lipids); EX1006 at 2-3 (same); EX1002 ¶¶6, 8 (contrasting lipoplexes and nucleic

acid-lipid particle).

97.   Lipoplexes and nucleic acid-lipid particles have different structures.

For example, liposomal bilayers form around encapsulated nucleic acids, thereby protecting the nucleic acids from degradation by environmental nucleases; lipoplexes, by contrast, do not encapsulate nucleic acids, and hence, cannot completely sequester them away from environmental nucleases. Moreover, liposomes can encapsulate, in their aqueous compartments, other bioactive agents in addition to nucleic acids; lipoplexes, by contrast, cannot because they do not encapsulate aqueous volume.

EX2007, 2:54-62; *see also* EX2028, 122:1-24 (acknowledging distinction between lipoplexes and nucleic acid-lipid particles).

98.   Lipoplexes are also functionally distinct and were known to be unsuitable for many applications. EX2007, 2:26-40 (explaining that "lipoplexes suffer from several major drawbacks when used in gene therapy, including low stability, high cytotoxicity, non-biodegradability, poor condensation and protection of DNA, serum sensitivity, large size and lack of tissue specificity."); EX1002, ¶6; EX1005 at 3315; EX1008 at 5-6.

99.   The '196 PCT explicitly distinguishes lipoplexes from the nucleic acid-lipid particles described therein. *Compare* EX1002, ¶6 ("Cationic liposome complexes, however, are large, poorly defined systems that are not suited for systemic applications and can elicit considerable toxic side effects.") *with id.*, ¶2 ("[T]he present invention is directed to using a small interfering RNA (siRNA)

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encapsulated in a serum-stable lipid particle having a small diameter suitable for systemic delivery.").

100.   Dr. Janoff asserts that a person of ordinary skill in the art "a POSITA would have found it obvious to use the insights of Lin regarding increasing the cationic mole fraction of nucleic acid lipid particles to increase transfection efficiency and the disclosures of the Patent Owner's prior disclosures regarding nucleic acid-lipid particles with a cationic lipid proportion greater than 50%." EX1007 ¶142. I disagree. Because of the differences between lipoplexes and the claimed nucleic acid-lipid particles, a person of ordinary skill in the art would not have found teachings regarding lipoplexes to be relevant for the development of nucleic acid-lipid particle compositions.

101.   Moreover, the petition materials falsely assert that "[a] POSITA would understand the testing of Ahmad to support the proposition that for certain formulations, cationic lipids can increase transfection efficiency when they are incorporated above 50 mol%." Pet. 49; EX1007 ¶139. But Ahmad discloses that transfection efficiencies for most lipoplexes are insensitive to cationic lipid increases over the range of 40 mol % to 80 mol % cationic lipid content. EX1006, Figure 3A. Similarly, Lin discloses that transfection efficiency for lipoplexes varies dramatically depending on which cationic lipid and non-cationic lipid are used. For example, the transfection efficiency of DOSPA/DOPC lipoplexes is

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insensitive over the range of 20 mol % to 100 mol % cationic lipid, EX1005,

Figure 4A, and DOTAP/DOPE lipoplexes are insensitive to cationic lipid increases

over the entire tested range (0 mol % to 70 mol %), EX1005 Figure 4D. If

anything, Lin supports the unpredictability of lipid particle chemistries.

Consequently, there is no teaching or suggestion in Lin or Ahmad to increase the

cationic lipid component above 50% is beneficial.

102.   Lin and Ahmad further provide that the cationic lipid component of

lipid particles should be minimized, regardless of whether used for in vitro or in

vivo purposes. For example, Ahmad specifically teaches that the cationic lipid

component should be minimized.

> A relatively low lipid/DNA charge ratio, therefore, can be considered
> optimal since it allows for achievement of maximum TE with the least
> amount of cationic lipid. This is due to the unexpected increase of σ_M*
> with ρchg. ***Minimizing the amount of cationic lipid is desirable to***
> ***reduce cost as well as potential toxic effects of the cationic lipid***. In
> addition, achieving a given σ_M with fewer, more highly charged
> molecules should mean a smaller metabolic effort for the elimination
> of the lipids from the cell.

EX1006 at 7 (emphasis added). This teaching is unequivocal and would not have

been ignored by a person of ordinary skill in the art.

103.   The Board in its institution decision stated that:

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We do recognize that Ahmad is concerned with the toxicity of
cationic lipids, but Ahmad noted that "with the amounts of cationic
lipid employed in our in vitro experiments, we find no toxic effects on
the cells as judged by cell morphology and the amount of total cellular
protein." Ex. 1006, 746. Because claim 1 is not limited to in vivo use
of the claimed nucleic acid-lipid particle, the statement in Ahmad to
which Patent Owner directs us concerning minimizing the amount of
cationic lipid to avoid cost and toxicity, is not necessarily persuasive
that Ahmad does not encourage increased amounts of cationic lipid in
certain circumstances.

Paper 15 at 31-32. I disagree. Ahmad states without qualification that cationic lipid

should be minimized to avoid toxicity. EX1006 at 7, 9. Moreover, Ahmad does not

qualify the conclusion that cationic lipid should be minimized even though no

toxicity was observed in a single particular set of her experiments.

104.   Furthermore, the toxicity assay is not described in sufficient detail to

meaningfully assess the results, and in any event, that in a single set of experiments

"toxic effects" were not observed does not take away from Ahmad's unequivocal

teaching or the teaching of other prior art references consistent with Ahmad. E.g.,

EX1008 at 5. Among other things, persons of ordinary skill in the art appreciated

that in vitro toxicity is dependent on how and when it is measured. For example, if

toxicity is measured too long after treatment with a toxic formulation, growth of

the surviving cells will give the false impression that the formulation is not toxic.

This is because surviving cells will continue to grow and, in time, replace those

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cells killed by the toxic formulation. A person of ordinary skill in the art would not ignore the problem of cytotoxicity based on a single unspecified observation to the contrary.

105.   In my opinion, as explained in further detail below, there would have been no good reason why a person of ordinary skill in the art would combine the different range disclosures for different lipid components from the '196 PCT so as to arrive at the claimed nucleic acid-lipid particle. Nor would one reasonably expect such formulations to work. The petition materials fail to demonstrate otherwise.

106.   The petition materials state that "given the success of generating nucleic acid-lipid particles with a cationic lipid proportion greater than 50% as described in the Patent Owner's prior disclosures, a POSITA would have appreciated a reasonable expectation of doing so." EX1007 ¶141. However, this statement is confusing to me because neither the '196 PCT nor the '189 publication disclose nucleic acid-lipid particles with cationic lipid proportion greater than 50%. EX1002 ¶¶ 216, 223, 228, 232 (disclosing formulations of 7.5 mol % and 15 mol % cationic lipid); EX1003 ¶¶289, 291-293, 295, 303, 311, 319, 327, 335, 343, 351, 361, 369, 377, 385 (disclosing formulations of 30 mol % and 40 mol % cationic lipid).

107.   Moreover, if anything, Lin and Ahmad strengthen the conclusion I arrived at in Ground 1 that a person of ordinary skill in the art would not have a reasonable expectation of success. For example, Ahmad expressly states that cationic lipid should be minimize to avoid toxicity and cost. EX1006 at 7.

IX.   GROUND 3 – CLAIMS 1-20 ARE NEITHER ANTICIPATED BY NOR OBVIOUS IN VIEW OF THE '554 PUBLICATION

108.   It is my opinion that the petition fails to demonstrate that claims 1-20 of the '435 patent are unpatenable in view of the '554 publication.

A.   Claim 1

1.   Anticipation by L054

109.   The '554 publication does not disclose the claimed nucleic acid-lipid particle composition, as claimed. First, L054 formulation of Table 4 is a lipid mixture for making particles, not a particle. Second, the petition fails to establish that L054-derived lipid particles would qualify as a nucleic-acid lipid particle as claimed, as there is no evidence that the L054-derived lipid particles encapsulate nucleic acid. Separately, the petition fails to establish that a L054-derived lipid particle of the '554 publication is suitable for systemic delivery as required by claim 1.

110.   The challenged claims are directed to a nucleic acid-lipid particle. The L054 lipid formulation identified in Table 4 of the '554 publication is a listing of lipid components or mixture of lipids used to form particles, not a nucleic acid-

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lipid particle as claimed. The petition materials confuse the composition of the input formulation (*i.e.*, lipids of Table 4) with something different—*i.e.*, the output formulation (*i.e.* lipid particles). There is no disclosure in the '554 publication regarding the composition of particles generated using the L054 formulation. This is an important distinction because a person of ordinary skill in the art would not assume that the composition of a lipid particle will be exactly the same as the composition of the lipid formulation utilized as a mixture of lipids to make the particles. Testing of the finished particle composition is necessary to account for variations in the molar fractions of the lipid components in the starting lipid formulation compared to the molar fractions of the lipid components in the resulting particles. EX2012 at 7242 ("Perhaps surprisingly, however, the composition of CLDCs is not usually determined at all—rather, the composition is simply assumed to be defined by the identity and amounts of the components originally introduced. In the future, however, as gene therapy agents approach pharmaceutical reality, more rigorous criteria likely will be required."); EX2013 (FDA guidance) at 3 (recommending labeling with the "amount of each lipid component used in the formulation based on the *final form* of the product" and "[a]n expression of the molar ratio of each individual lipid to the drug substance is also recommended for each individual lipid in the *finished formulation*), 8 (recommending reporting of "characteristics or attributes specific to the liposome

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formulation" including "[l]ipid content (*to demonstrate consistency with the intended formulation*).").

111. Experimental data presented in the '435 patent illustrates that the input and output formulations are not identical. For example, the '435 patent discloses that the lipid to drug ratio (*i.e.*, lipid to nucleic acid ratio) calculated from the input components is not identical to that of the finished product. *See, e.g.*, EX1001, 79:50-80:9 (reporting different input and final lipid to drug ratios for SNALP formulations). I understand that Dr. Janoff agrees with this assessment. *See* EX2028, 155:2-25 (indicating that there could be a difference between input and output lipid-to-drug ratio).

112. The '554 publication further evidences that the input molar ratio of lipids is not identical to that of the finished product. For example, the '554 publication discloses that lipid particles made from the lipid mixtures in Table IV (*e.g.*, L054) are "characterized in term of particle size, Zeta potential, alcohol content, total lipid content, nucleic acid encapsulated, and total nucleic acid concentration." EX1004 ¶634. That is, lipid and nucleic acid content and Zeta potential (*i.e.*, a measure of surface charge which relates to the amount of cationic lipid present in the particle) must be empirically determined and is not identical to the starting materials used to make the particles. *See* generally EX2013.

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113.   The methods disclosed in the '554 publication, including the identified detergent dialysis methods, would be expected to skew the molar ratio of lipids in the finished particles relative to the starting materials. For example, cholesterol-based detergents (*e.g.*, BIGCHAP and deoxy-BIGCHAP) are biased toward extracting cholesterol. *See* EX1004 ¶165 (listing BIGCHAP and deoxy-BIGCHAP as examples of suitable detergents for use with the detergent dialysis method of particle formation). The predictable result of using cholesterol-based detergents is less cholesterol in the finished particles than in the starting materials. When less cholesterol is incorporated into nanoparticles, the molar ratio of the remaining components (*i.e.*, cationic lipid, phospholipid, and conjugated lipid) is increased. I understand that Dr. Janoff agrees that cholesterol in the starting lipid mixture may not be quantitatively incorporated into finished particles. EX2028, 157:12-158:16 (explaining how failure to recover cholesterol in a particle would change the amount of the remaining components).

114.   A similar situation would apply to organic solvent-based methods. That is, differences in solubility in organic solvent amongst the lipid components of the mixture will result in differential incorporation of components into nanoparticles.

115.   In the L054 example, while the lipid formulation is listed as 50/20/28/2, the molar fractions of the same lipids in the resulting particle would be

expected be different and presumably outside the scope of the challenged claims. The L054 lipid mixture has cationic lipid content and conjugated lipid content that are at the edge of the claimed ranges. Because L054 has cationic lipid content and conjugated lipid content on the edge of the claimed range, even small differences in incorporation of components will result in lipid particles that are outside the claimed range of cationic lipid content and conjugated lipid content. For example, if cholesterol is not quantitatively incorporated, see above, particles derived from the L054 lipid mixture would have more than 2 mol % conjugated lipid.

116.   Accordingly, the '554 publication fails to disclose a lipid particle composition produced by the L054 lipid mixture that is within the scope of claim 1.

117.   The petition materials fail to establish that the L054-derived nanoparticles encapsulate nucleic acid, as is required by claim 1. As discussed above, the term "nucleic acid-lipid particles" excludes particles that do not encapsulate nucleic acid. The encapsulation state of the nucleic acids in nanoparticles made with the L054 lipid mixture is unknown.

118.   To the extent that the '554 publication discusses structure, it suggests that only serum-stable lamellar embodiments encapsulate nucleic acids.

> In one embodiment, the present invention provides a serum-stable formulated molecular composition … in which the biologically active molecule is encapsulated in a lipid bilayer and is protected from

degradation (for example, where the composition adopts a lamellar

structure).

EX1004 ¶136; *see also* ¶316. As discussed above, the L054 embodiment has not

been tested for serum stability and whether it adopts a lamellar structure that

encapsulates the nucleic acid is similarly unknown.

119.   Encapsulation of nucleic acids cannot be assumed based on the

composition and formulation method. I understand that Dr. Janoff agrees with me.

> However, neither of [the prior art] preparations were dialyzed against
>
> high salt buffers subsequent to liposome formation, the reported
>
> amounts of encapsulated DNA actually may include a significant
>
> percentage of unencapsulated DNA. Since [the prior art] liposomal
>
> formulations were not exposed to DNAase degradation to determine
>
> the percentage of DNA actually sequestered in the liposomes, the high
>
> reported amounts probably do not reflect actually encapsulated DNA.

EX2007, 4:11-19. The '554 publication does not disclose dialyzing nanoparticles

made from the L054 lipid mixture against high salt buffers or exposing the

preparation to DNAse degradation. Moreover, the '554 publication does not

disclose methods for determining encapsulation of nucleic acids.

120.   Moreover, as indicated above, Dr. Janoff opined that the lipid

particles of the '435 patent are SNALP. That is, the inventive lipid particles are

serum stable nucleic acid-lipid particle that are "extremely useful for systemic

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applications" and "can exhibit extended circulation lifetimes following intravenous (i.v.) injection." EX1001, 11:36-38.

121.   However, the petition and Dr. Janoff fail to address whether L054-derived nanoparticles are formulated for systemic administration. The '554 publication distinguishes between embodiments formulated for *in vitro* use and those formulated for *in vivo* use. *See, e.g.*, EX1004 ¶¶136, 462. Furthermore, the '554 publication stresses that serum-stability is a critical property of *in vivo* formulations. *See, e.g.*, EX1004 ¶¶14, 15, 158. However, L054 was only tested in vitro. *See* EX1004 ¶395 ("FIG. 16 shows a non-limiting example of ***in vitro efficacy*** of siNA nanoparticles …") (emphasis added). Specifically, the L054 formulation was not evaluated for serum stability — a property identified as critical for embodiments formulated for systemic (*in vivo*) use. *See* EX1004 ¶¶158 (providing a serum stability test "for determining whether a formulated molecular composition will be effective for delivery of a biologically active molecule into a biological system, …"), ¶592 (Example 7 Evaluation of Serum Stability of Formulated siNA Compositions). Furthermore, while another exemplary formulation (outside the scope of the '435 claims) was tested *in vivo*, the L054 formulation was not. EX1004 ¶596.

122.   Furthermore, a person of ordinary skill in the art would have expected particles derived from L054 to be too toxic for systemic use. Such a skilled artisan

would have expected DMOBA, the cationic lipid used in the L054 lipid mixture (see Table IV), to be toxic. A person of ordinary skill in the art would have appreciated that the dimethylamino group on the aryl ring is a good leaving group upon protonation and, as such, has the potential to alkylate cysteines and lysines on cellular proteins. *See* EX1004, Table IV (DMOBA structure). Additionally, DMOBA would have been expected to accumulate in liver and spleen because the arylether groups make elimination through mammalian detoxification pathways more difficult. The resulting accumulation and protein modifications would result in organ toxicity rendering lipid particles using DMOBA inappropriate for systemic use.

123. Accordingly, the petition materials fail to identify a nucleic acid-lipid particle within the scope of claim 1.

2.   Anticipation by ranges

124. The petition and Dr. Janoff assert that claim 1 is anticipated by ranges disclosed in the '554 publication However, the petition and Dr. Janoff fail to demonstrate that a person of ordinary skill in the art would have understood the separate disclosures of ranges for the various components to represent a single formulation. Moreover, I understand that anticipation by ranges requires a factual inquiry into whether the ranges are disclosed with "sufficient specificity." Such an inquiry is wholly absent from the petition and Dr. Janoff's declaration.

125.   To the extent that these ranges are discussed, there is no explanation in the '554 publication or Dr. Janoff's declaration to indicate how the disclosed ranges for the three different components would represent or be combined into a single embodiment.

126.   For example, Dr. Janoff points to three paragraphs in the '554 publication for disclosure of ranges of lipid components. *See* EX1007 ¶¶146, 150, 151. These paragraphs are reproduced in relevant part below.

> *In one embodiment*, the cationic lipid component …of a composition of invention comprises from about 2% to about 60%, from about 5% to about 45%, from about 5% to about 15%, or from about 40% to about 50% of the total lipid present in the formulation.

EX1004 ¶116 (emphasis added).

> *In one embodiment*, the neutral lipid component of a composition of the invention comprises from about 5% to about 90%, or from about 20% to about 85% of the total lipid present in the formulation.

EX1004 ¶313 (emphasis added).

> *In one embodiment*, the PEG conjugate … of a composition of the invention comprises from about 1% to about 20%, or from about 4% to about 15% of the total lipid present in the formulation.

EX1004 ¶118 (emphasis added).

127.   These paragraphs state that "*in one embodiment*" the cationic lipid component can span four different ranges. Further, "*in one embodiment*" the neutral lipid component can span two different ranges. Still further, "*in one*

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embodiment" the PEG conjugate can span two different ranges. There is no

explanation in the '554 publication or Dr. Janoff's declaration to indicate how

these eight different ranges for three different components would represent or be

combined into a single embodiment. Moreover, as with L054-derived particles, the

petition and Dr. Janoff fail to address whether any of the embodiments relied upon

are suitable for systemic administration.

128.   Also absent is sufficient demonstration that ranges or examples relied

upon in '554 publication disclose the claimed range for "a conjugated lipid that

inhibits aggregation of particles" (*i.e.*, claim element 1(d)). The petition relies on

paragraph ¶504 of the '554 publication to support that it is "desirable to include

other components" that serve to prevent aggregation. Pet. 55. The petition

materials, however, fail to demonstrate or explain which, if any, specific

embodiments in the '554 publication includes conjugated lipid such that

aggregation of particles is inhibited as claimed.

129.   Moreover, as with L054-derived particles, the petition and Dr. Janoff

fail to address whether any of the embodiments relied upon are suitable for

systemic administration.

130.   Furthermore, the petition and Dr. Janoff fail to demonstrate that a

person of ordinary skill in the art would have understood the separate disclosures

of ranges for the various components to represent a single formulation. For

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example, relevant to such an inquiry is an analysis as to the size of the prior art ranges and the differences between the prior art ranges and the claimed ranges. No such analysis was provided by Dr. Janoff — he merely asserts "[g]iven the breadth of the claimed range, these disclosures are sufficiently specific to anticipate the claimed range." EX1007 ¶¶148, 150, 151.

131.   Based on my review of the size of the prior art ranges and the extent of the differences between the prior art ranges and the claimed ranges, it is my opinion that there are considerable differences between the prior art and claimed ranges. The ranges disclosed by the '554 publication which Dr. Janoff cites are very broad, and thus the '554 publication fails to provide the required sufficient specificity to anticipate the lipid component ranges of claim 1.

132.   Therefore, in my opinion, the lipid component ranges of the '554 publication do not anticipate the lipid component ranges of the claimed nucleic acid-lipid particle composition at least because the ranges disclosed by the '554 publication which Dr. Janoff cites are very broad, the '554 publication and Dr. Janoff fail to indicate how these broad ranges arrive at the specific ranges recited in claim 1, and a person of ordinary skill in the art would not have understood the separate disclosures of ranges for the various components to represent a single formulation.

3.   Obvious over ranges

133.   The claimed nucleic acid-lipid particle composition is not obvious in view the lipid component ranges of the '554 publication. First, the '554 publication fails to disclose the combination of the lipid components of the claimed nucleic acid-liposome particle. Second, a skilled artisan would have had no reason to combine individualized lipid components in the '554 publication to arrive at the claimed nucleic acid-lipid particle. Third, the petition materials fail to address that a skilled artisan would have had no reasonable expectation of success that the claimed nucleic acid-lipid particle composition would be well-tolerated and efficacious. Finally, unexpected results further demonstrate the nonobvious nature of the claimed nucleic acid-lipid particle composition.

134.   To the extent that the petition and Dr. Janoff argue that the claims are obvious over the disclosed ranges of lipids in the '554 publication, this argument fails for the same reasons as discussed in Ground 1. That is, the petition materials rely on generic disclosure of ranges for individual lipid components that may be used in lipid particle compositions. But a person of ordinary skill in the art knew that the properties of lipid particle compositions (*e.g.*, cytotoxicity and efficacy) derive from the entire composition (*i.e.*, cationic, non-cationic, and conjugated lipids), rather than the individual lipid components. Given the interdependent nature of the components of the claimed nucleic acid-lipid particle composition,

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the petition's per-limitation approach to addressing the levels of cationic, non-cationic, and conjugated lipids is inadequate.

135.   Moreover, "a conjugated lipid that inhibits aggregation of particles" is required by the challenged claims (*i.e.*, claim element 1(d)). The petition materials, however, rely on paragraph ¶504 of the '554 publication for the proposition that it is "desirable to include other components" that serve to prevent particle aggregation. EX1007 ¶151. The petition materials, however, fail to demonstrate or explain which, if any, specific embodiments in the '554 publication includes a conjugated lipid such that aggregation of particles is inhibited as claimed.

136.   Lastly, it is my understanding that a claim is only obvious if a person of ordinary skill in the art would have had some motivation to modify the cited reference, and a reasonable expectation of success in doing so. Dr. Janoff does not discuss these aspects. However, it is my opinion that there would not have been any motivation to modify the cited disclosures of the '554 publication. As already discussed, the state of the art was unequivocal that a high level of cationic lipid should be avoided, and that much higher levels of conjugated lipids should be used. To the extent that Dr. Janoff is suggesting that a person of ordinary skill in the art could simply vary the concentrations of each component until they arrived at the claimed invention, I disagree. The field of art is unpredictable. Furthermore,

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there was no guidance whatsoever that would suggest to a skilled artisan to increase the cationic lipid levels while decreasing the conjugate lipid levels.

137. It is also my opinion that there would not have been any reasonable expectation of success. As discussed, a person of ordinary skill in the art would not have expected lipid particle formulations that departed so drastically from the instructions in the prior art to successfully exhibit any efficacy (*e.g.*, gene silencing). The expected result of using the claimed lipid particle formulations would have been little to no efficacy, accompanied by a host of unwanted side effects — *e.g.*, toxicity, *in vivo* aggregation, and immunogenicity

B. Claims 2-20

138. According to the petition, claims 1-20 are being challenged under anticipation and obviousness theories. However, only a handful of claims unambiguously identify both an anticipation and obvious theory.

Claim	Theory
2, 3, 5, 6, 9, 13-20	From these disclosures, a POSITA would appreciate that the claim limitation is expressly disclosed. EX1007 ¶¶153, 154, 156, 157, 161, 165-172.
4	[G]iven the explicit disclosure of overlapping ranges, this limitation is *prima facie* obvious. EX1007 ¶155.

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Claim	Theory
7	Given the breadth of the claimed range for the phospholipid, these disclosures are sufficiently specific to anticipate the claimed range. EX1007 ¶158. This limitation is *prima facie* obvious. EX1007 ¶159.
8	Given the breadth of the claimed range, these disclosures are sufficiently specific to anticipate the claimed range. Moreover, given the explicit disclosure of an encompassing range, this limitation is *prima facie* obvious. EX1007 ¶160.
10	Because one of the listed species of PEG-lipid conjugates is disclosed, this element is anticipated. EX1007 ¶162.
11	This limitation would have been obvious in view of the '554 publication in light of the knowledge of a POSITA. EX1007 ¶163.
12	For the reasons stated above, the '554 publication discloses this range with sufficient specificity to anticipate. In the alternative, this range is *prima facie* obvious given the overlapping range in the '554 publication. EX1007 ¶164.

139.   Additionally, most of the claims simply map disclosure without any explanation as to its significance. *See, e.g.*, EX1007 ¶160. Finally, the dependency of claims is largely ignored therefore it is challenging to determine what theory of unpatentability is actually being advanced. *See, e.g.*, EX1007 ¶160 (discussing

claim 8 which depends from claims 1 and 5). I have done my best to respond to

what seems to be the theory of unpatentability for each of the dependent claims.

> **Claim 4. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from 50 mol % to 65 mol % of the total lipid present in the particle.**

140.   Dr. Janoff presents no independent argument for claim 4, but rather

refers back to his discussion of claim 1. For the same reasons as claim 1, the

petition and Dr. Janoff fail to demonstrate the unpatentability of claim 4. Moreover,

claim 4 recites a narrower range of cationic lipid than claim 1 — a range that is

well represented by the tested range of cationic lipid and, in particular, nucleic

acid-lipid particle compositions with 57 mol % cationic lipid which are

unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of

the unexpected results and claim 4 is even stronger than it is for claim 1.

141.   Accordingly, the '554 publication neither anticipates nor makes

obvious claim 4.

> **Claim 5. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof.**

142.   Dr. Janoff appears to suggest that claim 5 is anticipated by the L054

formulation. EX1007 ¶156 (citing EX1004 Table IV). As I discussed above, L054

does not anticipate claim 1 and therefore cannot anticipate claim 5.

143.   To the extent that Dr. Janoff is asserting an anticipation theory based on disclosure in paragraphs 85 and 455, he provides no guidance for why a person of ordinary skill in the art would select a mixture of cholesterol and phospholipid from long lists of non-cationic lipids which include phospholipids, nonphosphorous containing lipids, and sterols.

144.   To the extent that Dr. Janoff is presenting an obviousness theory, I understand that to require a reason to combine these disclosures and a reasonable expectation of success — both of which are absent. Moreover, claim 5 requires a mixture of phospholipid and cholesterol such as that found in 1:57 formulations which are unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 5 is even stronger than it is for claim 1.

145.   Accordingly, the '554 publication neither anticipates nor makes obvious claim 5.

> **Claim 6. The nucleic acid-lipid particle of claim 5, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.**

146.   Dr. Janoff states that "a POSITA would appreciate that the claim limitation is expressly disclosed" in paragraph 85 of the '554 publication.

> Suitable neutral lipids include those comprising ***any of a variety of neutral uncharged, zwitterionic or anionic lipids*** capable of producing a stable complex. They are preferably neutral, although

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they can alternatively be positively or negatively charged. … [list of neutral lipids].

EX1004 ¶85. To the extent that Dr. Janoff is expressing an anticipation theory, I understand that picking and choosing claim elements from a prior art disclosure is not proper. To the extent that he is arguing that the claim is obvious, I understand that such a theory requires a reason to combine and reasonable expectation of success — both of which are absent.

147.   Accordingly, the '554 publication neither anticipates nor makes obvious claim 6.

> **Claim 7. The nucleic acid-lipid particle of claim 5, wherein the phospholipid comprises from 3 mol % to 15 mol % of the total lipid present in the particle.**

148.   Dr. Janoff appears to be alleging that claim 7 is anticipated or obvious over paragraph 455, which is a long list of neutral lipid components and generic ranges for neutral lipids and cholesterol disclosed in paragraphs 117-119 of the '554 publication. Dr. Janoff arrives at the "disclosure" of 3 mol % to 15 mol % phospholipid through a series of unexplained assumptions and mathematical manipulations. Dr. Janoff selects the 20% to 85% range of neutral lipid and the 20% to 45 % range of cholesterol from the paragraphs below.

> In one embodiment, the neutral lipid component of a composition of the invention comprises from about 5% to about 90%, or from about 20% to about 85% of the total lipid present in the formulation.

In one embodiment, the PEG conjugate (*i.e.*, PEG-DAG, PEG-cholesterol, PEG-DMB) of a composition of the invention comprises from about 1% to about 20%, or from about 4% to about 15% of the total lipid present in the formulation.

In one embodiment, the cholesterol component of a composition of the invention comprises from about 10% to about 60%, or from about 20% to about 45% of the total lipid present in the formulation.

EX1004 ¶¶117-119. Dr. Janoff then concludes:

When cholesterol is present, the range for a phospholipid is thus 0-40%. Not only does the disclosed range encompass the claimed range, when combined with a cationic lipid proportion in the 60% range and cholesterol in the 20-40% range, the range for the phospholipid is decreased to 0%-20%. Given the breadth of the claimed range for the phospholipid, these disclosures are sufficiently specific to anticipate the claimed range.

EX1007 ¶158. I do not understand this argument. There is just no basis to conclude that the '554 publication "discloses" a range of 0% to 40% or 0% to 20% phospholipid.

149.   Moreover, 1:57 nucleic acid-lipid particle compositions are encompassed by claim 7 and are unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 7 is even stronger than it is for claim 1.

150.   Accordingly, the '554 publication neither anticipates nor makes obvious claim 7.

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Claim 8. The nucleic acid-lipid particle of claim 5, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle.

151.   As an initial matter, claim 8 depends from claims 1 and 5. One of the anticipation theories presented in claim 5 was based on the L054 lipid mixture, which has 28 mol % cholesterol and therefore cannot be used to make particles with 30 mol % to 40 mol % cholesterol as recited in claim 8. The other anticipation theory in claim 5 was based on disclosure in paragraphs 85 and 455 which recite long lists of non-cationic lipids. I do not understand the relevance of paragraph 119, which recites ranges of cholesterol, to an anticipation theory. Among other reasons, this paragraph does not address the phospholipid content required by claim 5.

152.   Dr. Janoff also argues that paragraph 119 represents "an encompassing range" and therefore the claim is prima facie obvious. But again, claim 8 depends from claim 5 which requires a mixture of phospholipid and cholesterol. This "encompassing range" for cholesterol does not make any mention of phospholipid. Furthermore, a reason to combine these disclosures and reasonable expectation of success are absent from Dr. Janoff's analysis.

153.   Dr. Janoff also argues that "the '554 publication also includes various specific formulations which include cholesterol at a 30% proportion. Id., Table 4 (*e.g.*, L106)." First, L106 is a lipid mixture and not a nucleic acid-lipid particle.

Second, even if particles were made from L106, these particles would not meet the limitations of claim 8 which depends from claims 1 and 5. L106 has 3% conjugated lipid, which is well above the range of claim 1 (*i.e.*, 0.5% to 2%). Further L106 does not "comprise[] a mixture of a phospholipid and cholesterol or a derivative thereof" as required by claim 5. L106, as with the other examples from the '554 publication with 30% cholesterol, do not meet the limitations of claim 8.

154.   Moreover, 1:57 nucleic acid-lipid particle compositions are encompassed by claim 8 and are unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 8 is even stronger than it is for claim 1.

155.   Accordingly, the '554 publication neither anticipates nor makes obvious claim 8.

> **Claim 11. The nucleic acid-lipid particle of claim 10, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.**

156.   Dr. Janoff admits, that the '554 publication does not disclose a PEG-dialkyloxypropyl (PEG-DAA) conjugates as required in claim 11 and thus the '554 publication does not anticipate claim 11.

157.   Dr. Janoff alleges that "[a] POSITA would have been aware that PEG-dialkyloxypropyl (PEG-DAA) conjugates could be used in lieu of PEG-diacylglycerol (PEG-DAG) conjugates." EX1007 ¶163. Dr. Janoff points to

EX1014 as the "'910 publication" and states that it discloses PEG-DAA

conjugates. I do not know what EX1014 is, but it is not the '910 publication.

Regardless, Dr. Janoff's argument is based on the false assumption that PEG-DAG

and PEG-DAA conjugates are interchangeable. This is not the case. PEG-DAG

conjugates have an ester moiety linking the acyl chains (R^1 and R^2 in the figure) to

the backbone whereas PEG-DAA conjugates have an ether moiety linking the acyl

chains to the backbone. *See* EX1001, 53:53-54:14 (inset). As compared to PEG-

DAA conjugates, PEG-DAG conjugates are more hydrophilic which directly

impacts the physical properties of the particles. Moreover, PEG-DAG conjugates

are more easily metabolized by cells. A skilled artisan would not consider PEG-

DAG and PEG-DAA conjugates to be equivalent or interchangeable.

PEG-DAG   PEG-DAA
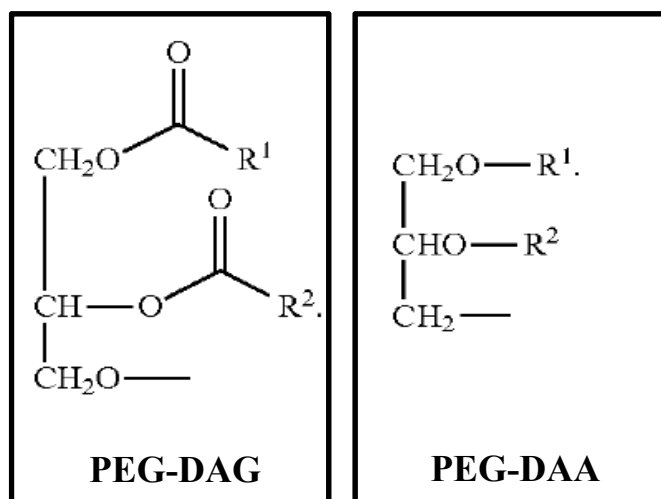
158.   Accordingly, the '554 publication neither anticipates nor makes

obvious claim 11.

Claim 12. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises from 1 mol % to 2 mol % of the total lipid present in the particle.

159.   Dr. Janoff presents no independent argument for claim 12, but rather refers back to his discussion of claim 1. For the same reasons as claim 1, the petition and Dr. Janoff fail to demonstrate the unpatentability of claim 12. Moreover, claim 12 recites a narrower range of cationic lipid than claim 1 — a range that is well represented by the tested range of cationic lipid and, in particular, nucleic acid-lipid particle composition with 57 mol % cationic lipid are unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 12 is even stronger than it is for claim 1.

Claim 13. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

160.   Dr. Janoff alleges that "a POSITA would understand that full encapsulation requires only an excess of cationic lipid with regard to the nucleic acid for electrostatic interaction." EX1007 ¶165. I disagree. Encapsulation is only determined by testing and cannot be inferred from the composition or production method. I understand that Dr. Janoff's shares my views. As he disclosed in one of his own patents:

> Additionally, [two prior art references] report encapsulation of 1-4 micrograms per micromole of spermine-condensed SV40 plasmid DNA in liposomes. However, neither of their preparations were dialyzed against high salt buffers subsequent to liposome formation,

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the reported amounts of encapsulated DNA actually may include a

significant percentage of unencapsulated DNA. Since these liposomal

formulations were not exposed to DNAase degradation to determine

the percentage of DNA actually sequestered in the liposomes, the high

reported amounts probably do not reflect actually encapsulated DNA.

EX2007 (Janoff) 4:15-19. Dr. Janoff's statements about full encapsulation are

remarkably similar to how the term is defined in the '435 patent.

The term "fully encapsulated" indicates that the active agent or

therapeutic agent in the lipid particle is not significantly degraded

after exposure to serum or a nuclease or protease assay that would

significantly degrade free DNA, RNA, or protein. In a fully

encapsulated system, preferably less than about 25% of the active

agent or therapeutic agent in the particle is degraded in a treatment

that would normally degrade 100% of free active agent or therapeutic

agent, more preferably less than about 10%, and most preferably less

than about 5% of the active agent or therapeutic agent in the particle is

degraded.

EX1001, 23:5-15. That is, full encapsulation requires a determination that the

nucleic acid is protected from nuclease degradation.

161.   Dr. Janoff does not point to a nuclease degradation assay in the '554

publication — because there is no such disclosure. Instead he alleges that

encapsulation finds support in paragraph 11.

The encapsulation of anionic compounds using cationic lipids is

essentially quantitative due to electrostatic interaction. In addition, it

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is believed that the cationic lipids interact with the negatively charged cell membranes initiating cellular membrane transport (Akhtar et al., 1992, Trends Cell Bio., 2, 139; Xu et al., 1996, Biochemistry 35, 5616).

EX1004 ¶11. This paragraph is describing prior art formulations and not embodiments of the '554 publication. Additionally, paragraph 11 is describing prior art cationic liposome-DNA complexes (*i.e.*, lipoplexes). I understand that Dr. Janoff asserts that lipoplexes do not encapsulate nucleic acids. EX2007, 2:54-59. ("[L]iposomal bilayers form around encapsulated nucleic acids, thereby protecting the nucleic acids from degradation by environmental nucleases; lipoplexes, by contrast, do not encapsulate nucleic acids, and hence, cannot completely sequester them away from environmental nucleases."). I do not understand why Dr. Janoff suggests paragraph 11 is relevant to the limitation of claim 13.

162.   Accordingly, the '554 publication neither anticipates nor makes obvious claim 13.

> **Claim 14. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier.**

163.   With respect to claim 14, Dr. Janoff points to disclosure of a pharmaceutical carrier and summarily concludes that this limitation is "expressly disclosed." EX1007 ¶162. As I discussed above, claim 1 is not anticipated under any theory nor are any claims depending from claim 1.

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JA00848

164.   To the extent that Dr. Janoff is presenting an obviousness theory, I

understand that it requires a reason to combine and reasonable expectation of

success — both of which are absent.

> **Claim 16. A method for the in vivo delivery of a nucleic acid, the method comprising: administering to a mammalian subject a nucleic acid-lipid particle of claim 1.**
>
> **Claim 17. A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising: administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 1.**
>
> **Claim 18. The method of claim 17, wherein the disease or disorder is a viral infection.**
>
> **Claim 19. The method of claim 17, wherein the disease or disorder is a liver disease or disorder.**
>
> **Claim 20. The method of claim 17, wherein the disease or disorder is cancer.**

165.   As I discussed above, the '554 publication neither anticipates nor

makes obvious the nucleic acid-lipid particles of claim 1. Therefore, any method

that claims the in vivo delivery of the nucleic acid-lipid particles of claim 1 is

similarly not anticipated and not obvious over the '554 publication.

166.   A person of ordinary skill in the art would not find it obvious to

administer the nanoparticles of the '554 publication to mammalian subjects in

vivo. This is because a skilled artisan would have expected the disclosed

nanoparticles to be too toxic for in vivo administration. More particularly, a skilled

artisan would have expected DMOBA and DMLBA (see Table IV) to be toxic. A

person of ordinary skill in the art would have appreciated that the dimethylamino group on the aryl ring is a good leaving group upon protonation and, as such, has potential to alkylate cysteines and lysines on cellular proteins. Additionally, DMOBA and DMLBA would have been expected to accumulate in liver and spleen because the arylether groups make elimination through mammalian detoxification pathways more difficult. The resulting accumulation and protein modifications would result in organ toxicity rendering lipid particles using DMOBA and DMLBA inappropriate for systemic use.

167. In sum, a person of ordinary skill in the art would not have been motivated to administer the nanoparticles of the '554 publication to mammalian subjects. And, consequently, claims 16-20 are not obvious.

X. OBJECTIVE INDICIA OF NONOBVIOUSNESS

168. The nucleic acid-lipid particle formulations of the '435 patent solved a long-felt need for compositions that could safely and effectively deliver nucleic acids to target cells of patients. Skilled artisans were skeptical that compositions having high levels of cationic lipid (*i.e.*, 50 mol % to 85 mol %) and low levels of conjugated lipid (*i.e.*, 0.5 mol % to 2 mol %) would be effective and well-tolerated when administered in vivo. The combination of effectiveness and low toxicity that characterizes the claimed compositions surprised many in the field, including me. Finally, the unique properties of the claimed nucleic acid-lipid particle

formulations solved the delivery problem that hindered the field of siRNA drugs. Onpattro™, a first in class siRNA drug was recently approved for use in the United States and Europe and is a nucleic acid-lipid particle composition within the scope of claim 1.

169.   My opinion that the challenged claims of the '435 patent are not obvious in view of the prior art, as set forth above, is not dependent on objective indicia of nonobviousness. However, such objective indicia further support the conclusion that the challenged claims would not have been obvious to a person of ordinary skill in the art at the time of the invention.

A.   Long-felt need

170.   In 1998, it was discovered that double-stranded RNA molecules could mediate a sequence-specific destruction of mRNA in the model organism, *C. elegans*. EX2011 (ACS) at 41. This phenomenon was dubbed RNA interference or RNAi. A few years later, RNAi was shown to work in mammalian cells but only if the size of the double-stranded RNA was limited to 21-23 nucleotides. The biomedical potential of targeted destruction mammalian mRNAs was immediately appreciated. Fire and Mello were awarded the Nobel Prize in Physiology in 2006 for their discovery of RNAi. EX2011 at 41.

171.   RNAi can be used to selectively disable mRNAs in mammalian cells and thereby silence expression of a protein (*e.g.*, an aberrant protein causing

disease). However, a major challenge in bringing RNAi to the clinic was delivery

of the small interfering ("siRNA") to cells. EX2011 at 38 ("[P]hysical delivery of

the drugs to diseased cells is extremely challenging."). In 2003, for example, Dr.

Phillip Sharp, Nobel Laureate and co-founder of Alnylam Pharmaceuticals, was

asked about the challenges that lie ahead for RNAi drugs he answered "Delivery,

delivery, delivery." EX2014 at 11. Dr. Phillip Zamore, co-Founder of Alnylam

Pharmaceuticals, shared Dr. Sharp's concern about delivery. EX2011 at 42.

172.   Delivery of RNAi to cells in vivo was widely regarded as a major

challenge in the field. For instance, Dr. Anastasia Khvorova, Director of Biology at

Dharmacon, acknowledged that the delivery systems developed prior to 2003 for

antisense-based nucleic acid drugs were not promising for delivery of RNA drugs.

> Khvorova believes that the medical benefits of RNAi will be huge if
> the delivery issues can be resolved. "But we've looked at a lot of the
> delivery methods that have been used for antisense, and so far I
> haven't been impressed," she says.

EX2014 at 11.

173.   The mid-2000's (*i.e.*, 2005-2008) saw dramatic growth and

investment in RNAi-based therapeutics. A number of large pharmaceutical

companies entered the RNAi space through acquisition of biotechnology

companies that owned the intellectual property rights to various siRNA molecules,

such as Merck's acquisition of Sirna Therapeutics. Yet, despite $2.5-3.5 billion in

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investment, no solution for the delivery problem had been found. EX2015 at 1. In fact, all four RNAi drugs in clinical trials at this time were naked RNA — that is, the siRNA was administered without a delivery vehicle. None of those four RNAi drugs in clinical trial were ultimately successful.

174. By 2008, the industry-wide failure to identify a solution to the delivery challenge resulted in waning confidence that RNAi could deliver on its therapeutic promise. The large pharmaceutical companies that had entered the RNAi space just a few years prior, began searching for delivery solutions by acquiring nascent delivery technologies and developing in-house delivery programs. EX2015 at 2 ("Big Pharma quickly realized the mistake of putting IP before enablement as they scrambled to scout for delivery technologies and found the majority of them not to live up to their claims."), 10 ("Big Pharma may also have relied too much on assurances by pure-play companies that delivery technologies were more mature than they really were."). Much of the delivery technologies identified around this time were inferior to Patent Owner's SNALP technology. EX2015 at 10 ("It is interesting for example that Roche's Factor VII patent application (WO 2010/055041) features Alnylam's 'lipidoid' technology for the rodent studies, but then switched to Tekmira's SNALP liposomes for the nonhuman primate part of the patent application.").

175.   Prior to the first publication of the '435 patent disclosure, Alan Sachs, leader of RNA Therapeutics at Merck, identified delivery as the challenge to successfully developing RNAi drugs.

> I have no doubt that RNAi, if it hasn't already, will absolutely demonstrate efficacy. It's an incredible drug. What's interesting about what we do is that the drug isn't the problem. It's the delivery of it.

EX2016 at 7.

176.   Dr. Sachs indicated that while there were a large number of delivery technologies available in mid-2009, few had been tested systemically in a relevant animal model.

> We have a graph we've disclosed which represents the number of opportunities we have looked at to do exactly what you describe, which is collaborate, particularly in the delivery space to advance this field. We are fully funded to do that, not just the evaluation, but the actual work. And what's really disappointing is that when you look at that graph, which is current as of mid-2009, there were 250-260 interesting opportunities, and there are really only two or three which have data that's valuable—meaning they have data from non-human primates.

EX2016 at 4. As explanation for the lack of viable delivery chemistries, Dr. Sachs elaborated on the nature of the challenge.

> There's a lot of hype, and there's a lot of ideas. But it's not a straightforward problem. Injecting something in the bloodstream, leading to something appearing in the cytoplasm in the RNA-silencing

JA00854

complex, there are a lot of black boxes between those two steps.
People who are entering the field start with a white paper. It's much
like people who started on targeted therapeutics years ago started with
a white paper. If it were so easy, one would have to describe why so
few examples exist. The same is true in the RNAi delivery process.
You can write down the steps. You can write down what you think
will happen. But then you have to put it in a 50-nanometer particle
that's safe and potent to deliver.

Id.

177.   The long-felt need for a siRNA delivery vehicle and the difficulty in
finding a solution is further exemplified by the effort and money that Patent Owner
invested into SNALP technology.

[I]t took Tekmira 500 person-years and over US$200M to turn a
single technology, SNALP, into the prolific drug development engine
it is today.

EX2015 at 8.

178.   Prior to the nucleic acid-lipid particles disclosed in the '435 patent,
there were no proven solutions to the delivery problem.

B.   Skepticism

179.   It was widely believed that cationic lipid content should be minimized
to avoid cytotoxicity, aggregation, and unwanted interactions with the immune
system and non-target cells.

180.   For example, Dr. Zamore of Alnylam, acknowledged that various cationic lipid formulations could successfully deliver siRNA to cells in vitro but stated that "I wouldn't want anyone injecting cationic lipids into my bloodstream." EX2011 at 42.

181.   Dr. Sachs of Merck also expressed skepticism as to the safety of the SNALP platform for delivery of siRNA.

> First are lipid-based delivery systems. At the time of our acquisition of Sirna, they had successfully shown lipid-based delivery to the liver. Initially, it was through a collaboration with what is now called [Vancouver, BC-based] Tekmira. That was really the leading standard for the area. Several [applications to begin clinical trials] have been filed with the FDA. We spent a lot of internal research money and time on novel lipids. ***The liability of that platform is absolutely its safety***.

EX2016 at 5.

C.   Unexpected Results

182.   It is surprising and entirely unexpected that the first FDA approved RNAi drug to meet the long-felt need for a delivery vehicle for RNAi drugs was one that does not minimize the cationic lipid component. Even more surprisingly the claimed formulations have a low level of conjugated lipid.

183.   Patent Owner's patents and publications that were published after the filing date of the '435 patent provide data for additional formulations within the scope of claim 1.

184.   For example, in U.S. Patent No. 8,236,943 (the "'943 patent," EX2017) tested several 1:57 formulations. Specifically, the '943 patent tested 1:57 formulations that used C2K, g-DLen-C2K-DMA, or DLen-C2K-DMA as the cationic lipid. Figure 7 depicts levels of gene silencing obtained after systemic administration of 0.010, 0.033, or 0.100 mg/kg of the various nucleic acid-lipid particle formulations. EX2017, 153:45-47.
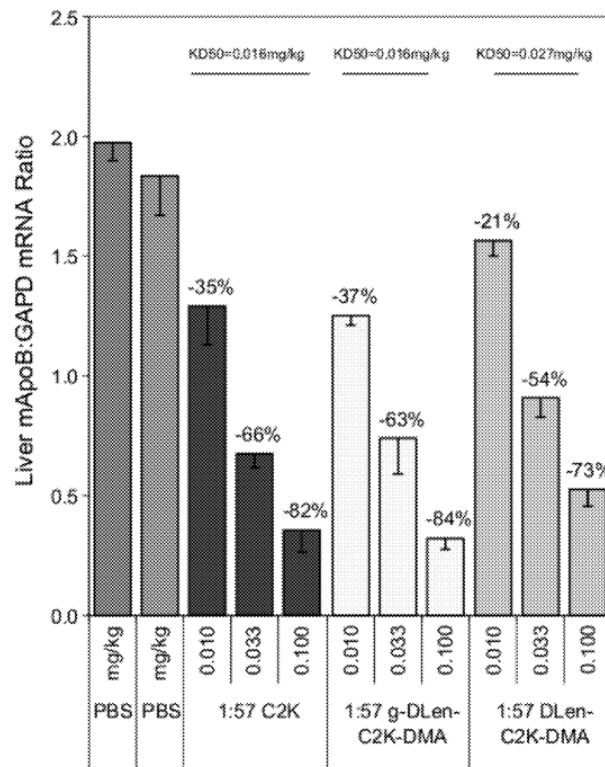
EX2017, Figure 7. Each formulation exhibited gene silencing activity far superior to that of the control PBS group. Further, the "SNALP formulation containing g-DLen-C2K-DMA displayed similar ApoB silencing activity at all three doses" and "the SNALP formulation containing DLen-C2K-DMA displayed considerable potency in silencing ApoB mRNA expression." EX2017, 153:45-55.

185.   U.S. Publication No. 2013/0116307 ("'307 publication," EX2018) also tested 1:57 formulations. Specifically, the '307 publication tested 1:57 formulations that used MC3, C2K, g-Len-MC3, CP-g-Len-MC3, Len-MC3, and CP-Len-MC3 as the cationic lipid.
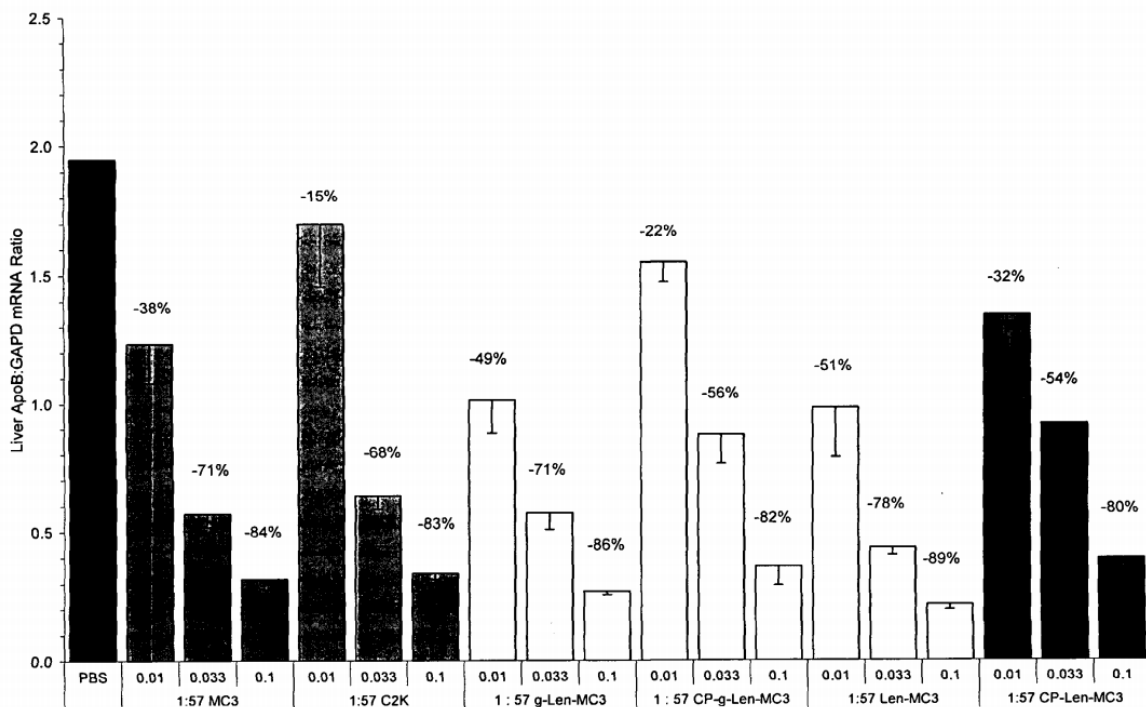
EX2018, Figure 5. Figure 5 depicts levels of gene silencing obtained after systemic administration of 0.010, 0.033, or 0.100 mg/kg of the various nucleic acid-lipid

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particle formulations. EX2018 ¶439. Each formulation exhibited gene silencing

activity far superior to that of the control PBS group. Further, the '307 publication

explains that "a SNALP formulation containing either CP-γ-LenMC3 or CP-

LenMC3 displayed similar ApoB silencing activity compared to a SNALP

formulation containing the C2K benchmark cationic lipid at all three doses."

EX2018 ¶439.

186. Yet more 1:57 formulations were disclosed in Semple et al., *Rational*

Design of Cationic Lipids for siRNA Delivery, 28 Nature Biotechnology 172-178

(2010) ("Semple," EX2021). Specifically, Semple tested 1:57 formulations that

used KC2 as the cationic lipid. EX2021 at 177. Semple measured gene silencing

following systemic administration in non-human primates. EX2021 at 174.
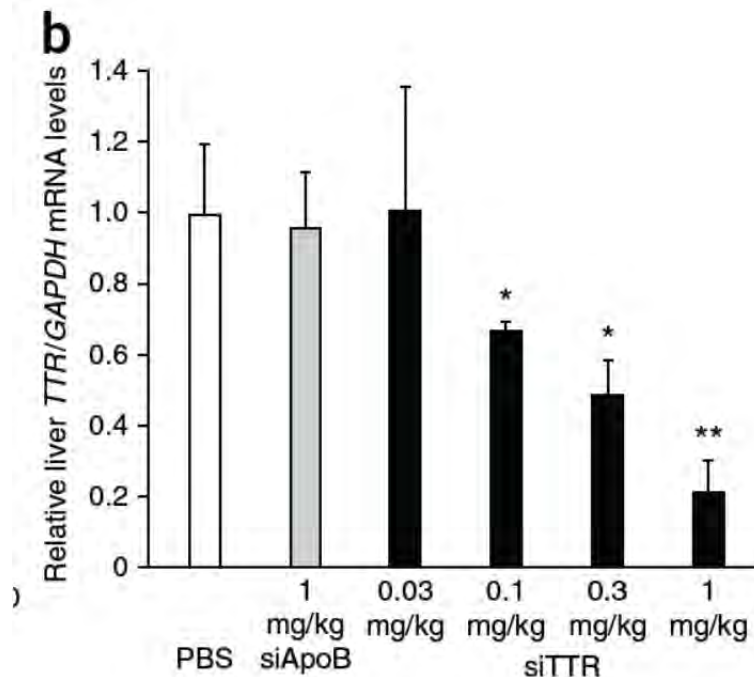
EX2021, Figure 3b. Semple explains that "[a] clear dose response was obtained with an apparent ED_{50} of ~0.3 mg/kg." EX2021 at 175. Further, "toxicological analysis indicated that the treatment was well tolerated at the dose levels tested, with no treatment-related changes in animal appearance or behavior." EX2021 at 175; *see also* id. ("Clinical signs were observed daily and body weights, serum chemistry and hematology parameters were measured 72 h after dosing. KC2-SNALP was very well tolerated at the high dose levels examined (relative to the observed ED_{50} dose) with no dose-dependent, clinically significant changes in key serum chemistry or hematology parameters."); EX2022 [Supplementary Materials Doc], Table 4.

187.   U.S. Publication No. 2017/0307608 to Bettencourt ("'608 publication," EX2019) discloses testing of a 1:50 formulation. The '608 publication is directed to the commercial product, Onpattro™ (*i.e.*, patisiran). The patisiran formulation is disclosed in Table 1. EX2019 ¶46, Table 1. Converting to mol % yields the following:

	DLin-MC3-DMA	PEG-c-DMG	DSPC	Cholesterol
mg	12.7	1.5	3.1	5.9
mol %	50	1.5	10	38.5

That is, patisiran is encompassed by claim 1.

188.   The '608 publication discloses the testing of patisiran in human subjects. As with the testing of 1:57 formulations in mice and non-human primates, patisiran was well tolerated in humans. EX2019 ¶103 ("The use of patisiran did not result in any significant changes in hematologic, liver, or renal measurements or in thyroid function, and there were no drug-related serious adverse events or any study-drug discontinuations because of adverse events."). Patisiran effectively silenced expression of its target — the TTR protein. EX2019 ¶121. ("[T]reatment of patients with FAP with patisiran led to robust, dose-dependent, and statistically significant knockdown of serum TTR protein levels."). The disclosed study found clinical benefit to treatment of patients with patisiran. EX2019 ¶132.

189.   A person of ordinary skill in the art would understand that the unexpected results of the '435 patent are not limited to formulations comprising specific ratios of components or comprising a specific type of cationic lipid. Specifically, between the '435 patent and subsequent publications, nucleic acid-lipid particle formulations with cationic lipid in the range of 50 mol % to 70 mol % and conjugated lipid in the range of 1.2 mol % to 2 mol % were tested and found to be efficacious and well tolerated. Additionally, nucleic acid-lipid particle formulations with eight different cationic lipids were tested and found to be efficacious and well tolerated. Finally, nucleic acid-lipid particle formulations within the scope of the claims were found efficacious and well tolerated in non-

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human primates and humans. These data span nearly the entire claimed ranges of cationic and conjugated lipid and are entirely unexpected.

D. Commercial Success

190.   The nucleic acid-lipid particles claimed by the '435 patent have achieved tremendous commercial success. Patisiran — tradename "Onpattro" — is a first in class siRNA drug. *See, e.g.*, EX2023 (Patisiran Nature News)at 291 ("US regulators have approved the first therapy based on RNA interference (RNAi), a technique that can be used to silence specific genes linked to disease."), ("'This approval is key for the RNAi field,' says James Cardia, head of business development at RXi Pharmaceuticals in Marlborough, Massachusetts, which is developing RNAi treatments. 'This is transformational.'"); EX2024 (FDA Release) ("FDA approves first-of-its kind targeted RNA-based therapy to treat a rare disease."); EX2025 (EMA Release) ("The European Medicines Agency's (EMA) Committee for Medicinal Products for Human Use (CHMP) has recommended granting a marketing authorisation for Onpattro (patisiran), …"). . Patisiran received regulatory approval in Europe on July 28, 2018 and the United States on August 10, 2018. *Id.*

191.   Patisiran was developed by Alnylam Pharmaceuticals under license from Arbutus. Specifically, Alnylam licenses the nucleic acid-lipid particle technology claimed in the '435 patent from Arbutus. EX2026 (Arbutus press

release). Under the license, Alnylam owes Arbutus royalties on the patisiran

product. *Id*.

192.   Patisiran is encompassed by claim 1 of the '435 patent. EX2019,

Table 1.

	Nucleic acid	Cationic lipid	Non-cationic lipid		Conjugated lipid
	siRNA-TTR	DLin-MC3-DMA	Cholesterol	DSPC	PEG-c-DMG
mg	2.0	12.7	5.9	3.1	1.5
mol %		50	38.5	10	1.5

193.   The '435 patent is one of the patents that encompasses the patisiran

commercial product. EX2027.

XI.   CONCLUDING STATEMENTS

194.   In signing this declaration, I understand that the declaration will be

filed as evidence in a contested case before the Patent Trial and Appeal Board of

the United States Patent and Trademark Office. I acknowledge that I may be

subject to cross-examination in this case and that cross-examination will take place

within the United States. If cross-examination is required of me, I will appear for

cross-examination within the United States during the time allotted for cross-

examination.

195.   I declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Dated: December 21, 2018   By: _____
                                                              David H. Thompson, Ph.D.

JOINT APPENDIX 30

**IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE**

ARBUTUS BIOPHARMA CORPORATION and GENEVANT SCIENCES GMBH *Plaintiffs*, v. MODERNA, INC. and MODERNATX, INC., *Defendants*.	C.A. No. 22-252-MSG
MODERNA, INC. and MODERNATX, INC., *Counterclaim-Plaintiffs,* v. ARBUTUS BIOPHARMA CORPORATION and GENEVANT SCIENCES GMBH *Counterclaim-Defendants*	**JURY TRIAL DEMANDED** **[**MODERNA'S CONFIDENTIAL INFORMATION REDACTED**]**

MODERNA'S INITIAL INVALIDITY CONTENTIONS

Pursuant to Paragraph 6(c) of the Court's March 21, 2023 Scheduling Order (D.I. 72),

Defendants and Counterclaim-Plaintiffs Moderna, Inc. and ModernaTX, Inc. (collectively

"Moderna" or "Defendants") hereby provide the following Initial Invalidity Contentions

("Contentions") with respect to the asserted claims of U.S. Patent Nos. 8,058,069 (the "'069

Patent"), 8,492,359 (the "'359 Patent"), 8,822,668 (the "'668 Patent"), 9,364,435 (the "'435

Patent"), 9,504,651 (the "'651 Patent"), and 11,141,378 (the "'378 Patent") (collectively, the

"Patents in Suit"), asserted by Plaintiffs and Counterclaim-Defendants Arbutus Biopharma

CONFIDENTIAL INFORMATION

1.   '651 Patent

a.   "fully encapsulated"

Claim 1 of the '651 patent is directed a "lipid vesicle formulation comprising . . . messenger RNA (mRNA) wherein at least 70% of the mRNA in the formulation is fully encapsulated in the lipid vesicle." All claims of the '651 patent depend, directly or indirectly, from claim 1.

The specification of the '651 patent discloses that "[a]s used herein, 'lipid encapsulated' can refer to a lipid formulation which provides a compound with full encapsulation, partial encapsulation, or both." '651 patent at 5:38-40. Aside from the claims, this is the only reference to "full encapsulation" or "fully encapsulated" in the '651 patent. In particular, nowhere in the specification of the '651 patent did the named inventors explain the meaning of "fully" encapsulated, as distinct from "partial[ly]" encapsulated. Nor does the specification teach a method to measure "fully encapsulated." A POSA would understand that the word "fully" does not refer to the proportion of the nucleic acid that is encapsulated (i.e. that 100% of the nucleic acid is encapsulated), because the proportion of nucleic acid that is encapsulated is recited elsewhere in the claim by a specific percentage ("at least 70[/80/90]% of the mRNA"). Because there is no indication in the specification as to the meaning of "fully encapsulated," a POSA would not understand the scope of the claim.

Further, even if a POSA in 2002 did understand the bounds of the term "fully encapsulated," which a POSA would not, the term is nonetheless indefinite as there is no method disclosed in the specification for measuring encapsulation, let alone distinguishing "fully" versus "partially" encapsulated nucleic acid.

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b.   **"wherein each lipid vesicle is a lipid-nucleic acid particle"**

To the extent Plaintiffs contend that the term "lipid-nucleic acid particle" requires stability, this term is indefinite, as it is not defined in the specification. A POSA in 2002 would have no guidance as to, *inter alia*, what type of stability is required, how to measure that stability and what degree of stability is required.

c.   **"wherein each lipid vesicle is a liposome"**

Plaintiffs have previously characterized as "***non*-liposomal**" the 1:57 SNALP described in the Molar Ratio Patents. *See* U.S. Patent 9,404,127. The Molar Ratio Patents describe the 1:57 SNALP as a "nucleic acid-lipid particle," and claim the invention is directed to "lipid-nucleic acid particle[s]." According to Plaintiffs, then, a "lipid-nucleic acid particle" is ***non*-liposomal**. Plaintiffs have asserted that Moderna's COVID-19 Vaccine infringes claims 8 and 9 of the '651 Patent, reciting a "liposome" and "lipid-nucleic acid particle" respectively, without explaining how Moderna's COVID-19 Vaccine meets either element. Plaintiffs also assert claims of the Molar Ratio Patents reciting "nucleic acid-lipid particles," without explaining how Moderna's COVID-19 Vaccine meets that element. Based on Plaintiffs' conflicting interpretation of these terms, the scope and meaning of the term "liposomal" is therefore uncertain, as a POSA would not know the characteristics required to meet the definition, rendering the term indefinite.

2.   **'069, '435, '359, '668, and '378 Patents (Molar Ratio Patents)**

a.   **"the total lipid present in the particle"**

Each independent claim of the Molar Ratio Patents is directed to a "nucleic acid-lipid particle" comprising or consisting essentially of certain lipids at specified mol% "of the total lipid present in the particle." During the '435 IPR and appeal, Arbutus (formerly Protiva) argued that it "cited extensive evidence demonstrating that the input formulation and the output formulation are not identical and that ***the finished particle must be tested to determine its final composition*** …

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CONFIDENTIAL INFORMATION

The evidence cited by Protiva clearly demonstrated that Moderna's anticipation challenge was based on the erroneous assumption that the composition of a particle is the same as the lipid mixture used to produce the particle." CAFC-20-1184, Dkt. 67-1 at 29 (emphasis added). In other words, Arbutus argued that the claims recite characteristics of the finished product.

The Molar Ratio Patents' specification discloses the mol% in the starting materials, as compared to the final nucleic acid-lipid particle:

TABLE 2

Characteristics of the SNALP formulations used in this study.

Sample No.	Formulation Composition, Mole % PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol	Lipid/Drug Ratio	Size (nm)	Polydispersity	% Encapsulation
			Finished Product Characterization		
1	2 | 40 | 10 | 48	12.4	57	0.07	90
2	1.8 | 36.4 | 18.2 | 43.6	14.0	72	0.12	89
3	1.4 | 27.0 | 6.8 | 64.9	16.5	70	0.12	92
4	1.3 | 25.3 | 12.7 | 60.8	18.1	76	0.07	93
5	3.9 | 39.2 | 9.8 | 47.1	13.5	53	0.27	86
6	3.6 | 35.7 | 17.9 | 42.9	15.1	58	0.18	87
7	2.7 | 26.7 | 6.7 | 64.0	17.6	56	0.17	92
8	2.5 | 25.0 | 12.5 | 60.0	19.2	61	0.13	92
9	1.4 | 57.1 | 7.1 | 34.3	17.8	84	0.10	88
10	1.3 | 53.3 | 13.3 | 32.0	19.5	83	0.10	89
11	1.1 | 42.6 | 5.3 | 51.1	22.0	80	0.10	93
12	1.0 | 40.4 | 10.1 | 48.5	23.6	78	0.11	88
13	2.8 | 56.3 | 7.0 | 33.8	19.0	62	0.14	80
14	2.6 | 52.6 | 13.2 | 31.6	20.6	66	0.14	82
15	2.1 | 42.1 | 5.3 | 50.5	23.1	71	0.16	91
16	2 | 40 | 10 | 48	24.7	67	0.14	92

'069 patent at Table 2. The specification of the Molar Ratio Patents further refers to the disclosed formulations as "target" formulations. *See, e.g.,* '378 Patent 25:55 ("It should be understood that these SNALP formulations are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the SNALP formulations may vary."); *id.* at 70:32-43 ("It should be understood that the 1:57 formulation and 1:62 formulation are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the formulation may vary. Typically, in the 1:57 formulation, the amount of cationic lipid will be 57 mol %±5 mol %, and the amount of lipid

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conjugate will be 1.5 mol%±0.5 mol %, with the balance of the 1:57 formulation being made up of non-cationic lipid (e.g., phospholipid, cholesterol, or a mixture of the two).”); *see also* ’378 patent at 71:16-77:22, Tables 2, 4, 6 and 7. The inventors therefore distinguished between starting composition and finished product characteristics.

If, contrary to positions asserted before the PTAB and Federal Circuit, Plaintiffs now attempt to take the position that the claims do not refer to the characteristics of the finished product, the claims are indefinite as POSA reading the claims in light of the specification in 2008 would fail to understand at what point the mol% of each of the claimed lipid components should be measured to determine whether a given composition falls within the scope of the claims.

Additionally, the claims of the Molar Ratio Patents encompass nucleic acid-lipid particles comprising or consisting essentially of three to four lipids and nucleic acids, providing ranges for the mole percentages of the claimed lipid types. However, the Federal Circuit in CAFC No. 20-2329 reasoned that “this case is not” “as simple as arbitrarily setting maximums and minimums for each individual component,” based on Arbutus’s arguments that this “because the lipid components of the nucleic acid-lipid particle are interdependent, and they interact with each other unpredictably.” CAFC No. 20-2329, Dkt. 66 at 15. According to Plaintiffs, the specification of the Molar Ratio Patents fails to adequately explain how a POSA in 2008 is to determine—or pick from the claimed ranges—the appropriate amount of each lipid to create a specific formulation. In other words, the claims are indefinite because the specification fails to explain which combinations of the mole percentages are claimed by these patents.

For at least these reasons, the claims of the Molar Ratio Patents are therefore invalid as indefinite.

JOINT APPENDIX 31

CHAPTER 9

Liposomal Formulations for Nucleic Acid Delivery

Ian MacLachlan

CONTENTS

9.1 LIPOSOMES FOR THE DELIVERY OF NUCLEIC ACID DRUGS

Liposomes are artificial vesicles made up of one or more bilayers of amphipathic lipid encapsulating an equal number of internal aqueous compartments. They are distinguished on the basis of their size and the number and arrangement of their constituent lipid bilayers (Figure 9.1).

Multilamellar   Small unilamellar   Large unilamellar
vesicle (MLV)   vesicle (SUV)   vesicle (LUV)
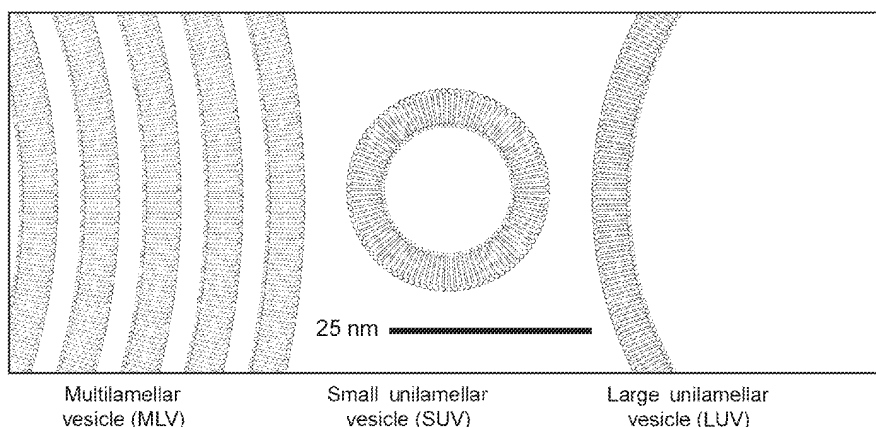
Figure 9.1 Liposomes. Multilamellar vesicles (MLVs) are large (hundreds of nm in diameter) complex structures containing a series of concentric bilayers separated by narrow aqueous compartments. Large unilamellar vesicles (LUVs) are between 50 and 500 nm in diameter, while the smallest liposomes namely small unilamellar vesicles (SUVs) are <50 nm. LUVs are the preferred systems for delivery of NA drugs. Lipids are drawn roughly to scale.

Multilamellar vesicles (MLVs) are formed by the aqueous hydration of dried lipid films. Typically hundreds of nanometers in diameter, they are large, complex structures containing a series of concentric bilayers separated by narrow aqueous compartments. Simple unilamellar vesicles between 50 and 500 nm in diameter are referred to as large unilamellar vesicles (LUVs) while the smallest liposomes, vesicles smaller than 50 nm in diameter, are small unilamellar vesicles (SUVs).

 Liposomes have received attention not only for their utility as model membrane systems, but also for use in drug delivery. Typically, liposomes are used as drug carriers, with the solubilized drug encapsulated in the internal aqueous space formed by the liposomal lamellae. Liposomal drug formulations can be used to overcome a drug's nonideal properties, such as limited solubility, serum stability, circulation half-life, biodistribution, and target tissue selectivity. Experience with conventional small molecule drugs has shown that the drugs which benefit the most from liposomal delivery, are those that are chemically labile, subject to enzymatic degradation and have an intracellular site of action [1]. For this reason, there is considerable interest in exploiting liposomes as carriers of nucleic acids (NAs), either as plasmid vectors for gene therapy applications or to deliver smaller NA species such as antisense oligonucleotides, ribozymes and, more recently, siRNA for the purposes of downregulating target genes. Because of their ability to achieve favorable drug/lipid ratios and their more predictable drug release kinetics LUV are the preferred liposome delivery system for NA drugs.

 An advantage of liposomal drug delivery is that the pharmacokinetics, biodistribution, and intracellular delivery of the liposome payload are largely determined by the physicochemical properties of the carrier. For example, the biodistribution of a NA entrapped within a small, long circulating liposome is independent of the type of NA, which can be a relatively stable double-stranded plasmid DNA molecule or single-stranded antisense DNA, or one of the more labile ribonucleotide molecules such as ribozymes or a duplex siRNA. This is only true if the liposome is truly acting as a carrier, rather than a mere excipient. Liposomes function as excipients when used to formulate hydrophobic drugs that would otherwise be difficult to administer in aqueous dosage form. Hydrophobic drugs rapidly exchange into lipoproteins or other lipid-rich environments soon after injection, resulting in comparably uncontrolled pharmacology. In the context of NA drug delivery, liposomes are considered excipients if used to enable vialing and aqueous dosing of hydrophobic lipid–NA conjugates [2–5]. (These applications are not considered in this chapter, nor are those that use preformed, cationic lipid-containing vesicles to form "lipoplex" or "oligoplex" systems.)

An objective inherent in all pharmaceutical development is to minimize the risks associated with treatment while maximizing the benefit to patient health. The most important risk to patients is the toxicity associated with the administration of poorly tolerated compounds, often exacerbated by attempts to increase efficacy by escalating the administered dose. A well-designed liposomal delivery system will be capable of reducing the toxicity and increasing the potency of NA-based drugs by optimizing NA delivery to target tissues. Liposomal NA delivery will be determined by the physical and biochemical properties of the liposome including stability, size, charge, hydrophobicity, interaction with serum proteins, and interaction with nontarget cell surfaces. Ideally, liposomal carriers for NA delivery will have the following properties: (i) they will be safe and well tolerated; (ii) they will have appropriate pharmacokinetic attributes to ensure delivery to intended disease sites; (iii) they will mediate effective intracellular delivery of intact NA; (iv) they will be nonimmunogenic, enabling the use of multidosing treatment regimes; and (v) they will be stable upon manufacture so that large batches can be prepared with uniform, reproducible specifications. In this chapter we discuss the physical makeup, manufacturing methods, and pharmacological considerations specific to liposomal systems for the delivery of NA-based drugs, with emphasis on those that enable systemic delivery of synthetic polynucleotides such as antisense ODN, ribozymes, and siRNA.

9.2 LIPOSOME CONSTITUENTS

NA encapsulation was first described in the late 1970s, prior to the development of cationic lipid-containing lipoplex, using naturally occurring, neutral lipids to encapsulate high-molecular-weight DNA [6–8]. The first reports of low-molecular-weight oligo- or polynucleotide encapsulation similarly used passive techniques to entrap NA in neutral liposomes [9–11]. The advent of cationic lipid-mediated lipofection [12] saw a shift in emphasis away from encapsulated systems in favor of "lipoplex" or "oligoplex" systems. More recently, improvements in formulation technology have allowed for a return to encapsulated systems that contain cationic lipids as a means of facilitating both encapsulation and intracellular delivery. More advanced systems typically contain multiple lipid components, each of which play a role in determining the physical and pharmacological properties of the system as a whole.

9.2.1 Cationic Lipids

Cationic lipids play two roles in liposomal NA formulations. In the first case, they encourage interaction between the lipid bilayer and the negatively charged NA, allowing for the enrichment of NA concentrations over and above that which would be achieved using passive loading in charge neutral liposomes. Cationic lipids allow for encapsulation efficiencies greater than 40% when using coextrusion methods, and greater than 95% when using more sophisticated techniques [13–15]. Cationic lipids also function by providing the liposome with a net positive charge, which in turn enables binding of the NA complex to anionic cell surface molecules. The most abundant anionic cell surface molecules, sulfated proteoglycans and sialic acids, interact with and are responsible for the uptake of cationic liposomes [16–18]. The role of cationic lipids in liposomal uptake presents a dilemma: highly charged systems are rapidly cleared from the blood, thereby limiting accumulation in target tissues. Particles with a neutral charge however, display good biodistribution profiles, but are poorly internalized by cells. This supports the concept of a modular delivery solution, that is, an engineered nanoparticle with individual components fulfilling different functions in the delivery process, and in particular, a system which responds to the microenvironment in a manner that facilitates transfection. Titratable, ionizable lipids are components that allow for the adjustment of the charge on the system by simply changing the pH after encapsulation [19]. At reduced pH when the system is strongly charged, NAs are efficiently encapsulated. When liposomes containing titratable, ionizable lipids are at a pH closer to the pK_a of the cationic lipid, such as

physiological pH, they become more charge neutral and are able to avoid opsonization by blood components [19]. More recently, the use of novel, pH titratable cationic lipids with distinct physicochemical properties that regulate particle formation, cellular uptake, fusogenicity, and endosomal release of NA drugs have been described [20]. The chemical and biological properties of pH-titratable cationic lipids are influenced by their degree of lipid saturation. In particular, the phase transition properties, as measured using ^{31}P-NMR, are affected. Above the phase transition temperature, T_c, lipids adopt the more highly fusogenic reverse hexagonal H_{II} phase [20–22]. By noting the temperature at which this phase transition occurs, the relative ease with which lipids form the H_{II} phase and become "fusogenic" can be determined. On this basis it has been shown that the fusogenicity of liposomal systems increases as the titratable cationic lipid becomes less saturated. The lipid pK_a also correlates with the degree of saturation. pK measurements confirm that saturated lipids carry more residual charge at physiological pH. For this reason, liposomes containing the more highly saturated cationic lipids are taken up more readily by cells in vitro [20]. However, liposomes containing the more fusogenic unsaturated cationic lipids DLinDMA and DLenDMA are more effective at mediating RNA interference in both in vitro cell culture systems and in vivo. The apparently conflicting results between cellular uptake and silencing potency are a reminder that cellular uptake per se is insufficient for effective delivery of NA. Cellular uptake, fusogenicity, and endosomal release are distinct processes, each of which need to be enabled by the delivery vehicle and each of which are profoundly affected by the physicochemical properties of the cationic lipids used.

9.2.2   The Role of Helper Lipids in Promoting Intracellular Delivery

Although we have just shown that cationic lipids may have inherent fusogenic properties of their own, cationic lipids were originally believed to require fusogenic "helper" lipids for efficient NA delivery [23–26]. Fusogenic liposomes facilitate the intracellular delivery of complexed plasmid DNA by fusing with the membranes of the target cell. Fusion may occur at a number of different stages in delivery, either at the plasma membrane, endosome or nuclear envelope. Fusion of first-generation, nonencapsulated lipoplex systems with the plasma membrane is expected to be a particularly inefficient method of introducing NA into the cytosol. Since lipoplex-NA is predominantly attached to the surface of the liposome, lipoplex fusion events resolve with NA, formerly attached to the liposome surface, deposited on the outside surface of the plasma membrane. Encapsulated systems are significantly different from lipoplex in this respect. Upon fusion with either the plasma or endosomal membrane(s), encapsulated carriers deliver their contents directly into the cytosol.

Lipids that preferentially form nonbilayer phases, in particular the reverse hexagonal H_{II} phase, such as the unsaturated phosphatidylethanolamine DOPE, promote destabilization of the lipid bilayer and fusion. Similar to fusogenic cationic lipids, decreasing the degree of lipid saturation increases the lipid's affinity for the fusogenic H_{II} phase [27–32]. However, some cationic lipids can function in the absence of these so-called helper lipids, either alone [24,25] or in the presence of the nonfusogenic lipid cholesterol [33]. This would suggest that either these lipids have properties which promote delivery through a mechanism which does not require membrane fusion, or that their own fusogenic properties are adequate to support delivery. As described above, cationic lipids are readily designed for optimal fusogenicity by controlling lipid saturation. This provides for multiple opportunities for modulating the fusogenicity of a liposomal lipid bilayer [20].

Attempts to address the role of fusogenic lipids in vivo have yielded confounding results. In this regard it is important to distinguish the effect of fusogenic lipids on NA delivery to target tissue from their effect on intracellular delivery. Fusogenic formulations are more likely to interact with the vascular endothelium, blood cells, lipoproteins, and other nontarget systems while in the blood compartment. For this reason there may be an advantage to transiently shield the fusogenic potential of systemic carriers using shielding agents such as polyethylene glycol (PEG).

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9.2.3 PEG–Lipids

An ideal delivery system would be one that is transiently shielded upon administration, facilitating delivery to the target site, yet becomes increasingly charged and fusogenic as it reaches the target cell. PEG lipids partially address this challenge. PEG–lipid conjugates are readily incorporated in liposomal NA formulations. They provide a benefit during the formulation process, stabilizing the nascent particle and contribute to formulation stability by preventing aggregation in the vial [13]. PEG conjugates sterically stabilize liposomes by forming a protective hydrophilic layer that shields the hydrophobic lipid layer. By shielding the liposome's surface charge they prevent the association of serum proteins and resulting uptake by the reticuloendothelial system when liposomes are administered in vivo [34,35]. In this way, cationic liposome NA formulations are stabilized in a manner analogous to PEGylated liposomal drug formulations that exhibit extended circulation lifetimes [36–41]. Although this approach has been investigated with a view towards improving the stability and pharmacokinetics of lipoplex containing either plasmid DNA [42] or antisense oligonucleotides [43], PEG–lipid-containing lipoplex systems suffer from the heterogeneity and suboptimal pharmacology common to most nonencapsulated NA–cationic lipid complexes.

Although PEG–lipid-containing systems are promising with respect to their ability to deliver NA to disease sites, improvements are required to increase their potency. Early PEGylated liposomes for the delivery of small molecule chemotherapeutic drugs utilized stably integrated PEG lipids such as PEG-DSPE [39]. These systems are designed to function as carriers that facilitate the accumulation of active drug compound at disseminated disease sites. The drug is released at the cell surface at a "leakage rate" determined by the liposomal bilayer composition. NA-based drugs differ in this respect in that they require effective *intracellular* delivery, hence the use of the cationic and fusogenic lipids described earlier. PEGylated systems typically exhibit relatively low-transfection efficiencies. This is mainly due to the ability of the PEG coating to inhibit cell association and uptake [23,44,45]. Ideally, PEG–lipid conjugates would have the ability to dissociate from the carrier and transform it from a stable, stealthy particle to a transfection-competent entity at the target site. Various strategies have been applied to this problem. A number of investigators have explored the use of chemically labile PEG–lipid conjugates [46–52], in particular those that are "pH sensitive." Typically, these systems invoke a chemically labile linkage between the lipid and PEG moieties that reacts via acid-catalyzed hydrolysis to destabilize the liposomes by removal of the sterically stabilizing PEG layer. Although this approach results in improved performance both in vitro and in vivo, it may be regarded as suboptimal for two reasons. First, pH-sensitive PEG lipids are designed to be rapidly hydrolyzed in the reduced pH environment encountered within the endosome, but since PEG lipids are known to inhibit cellular uptake, a prerequisite to endosomal localization and hydrolysis, their use actually limits the amount of material delivered to the endosome [53]. Second, the incorporation of pH-sensitive or otherwise chemically labile lipids results in a truncation of formulation shelf life relative to systems that use more stable PEG–lipids. An alternative to the use of acid-labile PEG–lipids involves the use of chemically stable, yet diffusible PEG lipids.

The concept of diffusible PEG lipids arose from the observation that the length of the PEG lipid anchor has an influence on PEG lipid retention and the stability and circulation lifetime of empty lipid vesicles [54]. It has been found that by modulating the alkyl chain length of the PEG lipid anchor [55–59], the pharmacology of encapsulated NA can be controlled or "programmed" in a predictable manner. Upon formulation, the liposome contains a full complement of PEG in steady-state equilibrium with the contents of the vial. In the blood compartment, this equilibrium shifts and the PEG–lipid conjugate is free to dissociate from the particle over time, revealing a positively charged and increasingly fusogenic lipid bilayer that transforms the particle into a transfection-competent entity. Diffusible PEG lipids differing in the length of the their lipid anchors have been incorporated into liposomal systems containing plasmid DNA (SPLP) [13,55], antisense oligonucleotides (PFV, SALP) [19,56,60], and siRNA (SNALP) [14,15,61]. This approach may help to resolve the two conflicting demands imposed upon NA carriers. First, the carrier must be stable and circulate long

enough to facilitate accumulation at disease sites. Second, the carrier must be capable of interacting with target cells to facilitate intracellular delivery.

9.2.4   Active Targeting

Active targeting refers to processes that aim to increase the accumulation, retention or internalization of a drug through the use of cell-specific ligands. This is to be distinguished from the passive "disease site targeting" or the "enhanced permeability and retention" (EPR) effect, which results in the accumulation of appropriately designed carriers in target sites such as tumor tissue. Active targeting has been successfully applied to liposomal small molecule drug formulations and generally has the effect of improving the therapeutic index of the liposomal drug when measured in preclinical studies. NA delivery systems stand to benefit from targeting in two ways, first through improving the accumulation and binding of formulations to target cells and second by facilitating intracellular delivery through endocytosis. The perceived benefits of active targeting have encouraged numerous investigators in this area and targeting of NA formulations has been achieved through the use of molecules as diverse as antibodies directed against cell surface proteins [62–65], protein ligands of cell surface receptors [66–69], vitamins [70–72], and glycolipids [73,74].

The earliest reports of targeted liposomal formulations of encapsulated NA were attempts to improve the intracellular delivery characteristics of charge neutral liposomes encapsulating either synthetic antisense DNA [63,65] or in vitro transcribed antisense RNA [64]. The results of these studies were encouraging, suggesting a significant benefit associated with the use of targeted systems. Although these in vitro studies effectively demonstrated the potential advantage of targeting at the level of intracellular delivery, they were unable to address important pharmacological considerations such as those that influence accumulation at disease sites. It is unlikely that addition of targeting ligands to delivery systems that are rapidly removed from the circulation will result in delivery exceeding that achieved by systems that display passive disease site targeting. For this reason many investigators have pursued approaches involving the addition of targeting ligands to sterically stabilized and charge shielded systems, such as those containing PEG lipids [71,72,75–77]. This approach has been advanced, in part, by the development of the so-called postinsertion technique [78]. Postinsertion allows for the insertion of ligand–PEG–lipid conjugates into preformed liposomes containing encapsulated NA. This represents a significant improvement on earlier approaches in which ligands were chemically coupled to preformed liposomes, an approach limited by suboptimal coupling efficiencies, or where ligand–lipid conjugates were incorporated in the first stages of the formulation process, an approach limited by the resulting negative impact on NA encapsulation efficiency and subsequent suboptimal presentation of the targeting ligand.

A number of reports suggest that it is possible to design encapsulated systems containing targeting ligands that retain extended circulation lifetimes and passive disease site targeting the following systemic administration. It remains to be seen if the benefits of active targeting outweigh the increased cost, manufacturing complexity and immunogenicity that often accompanies the use of such technology.

9.3   METHODS OF ENCAPSULATING NUCLEIC ACIDS

To capitalize on the pharmacology of liposomal drug carriers it is necessary to completely entrap NA within the contents of a liposome. In this regard it is important to distinguish first-generation "lipoplex" or "oligoplex" systems from those that truly encapsulate their NA payload. Lipoplex are electrostatic complexes formed by mixing preformed cationic lipid-containing vesicles with NA [12,79,80]. The result is a heterogenous, metastable aggregate that is effective when used to transfect cells in culture but has relatively poor performance in vivo. Upon systemic administration, lipoplex systems are rapidly cleared from the blood, accumulating in the capillary bed of first-pass organs such as the lung.

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Lipoplex are effectively taken up by the cells of the innate immune system, contributing to their profound toxicities and off-target effects. These side effects may manifest as "efficacy" in antitumor or anti-infective applications, confounding data interpretation and encouraging the acceptance of false-positive results. For these reasons, an abundance of caution is encouraged when initiating in vivo studies that use liposomes to deliver NA. Of particular importance is the use of appropriate analytical methodology, described in Section 9.4, to properly characterize lipid-based systems prior to and during use.

9.3.1 Passive Nucleic Acid Encapsulation

Liposomal encapsulation of small molecule drugs may be achieved by either "passive" or "active" loading. Unlike small molecule drugs, NAs are not readily packaged in preformed liposomes using pH gradients or other similar active loading techniques. This is predominantly due to the large size and hydrophilic nature of NA, which conspire to prevent them from crossing intact lipid bilayers. For this reason, much of the work on NA encapsulation has utilized passive loading technology.

Passive encapsulation typically involves the preparation of a "lipid film," the lipidic residue that remains after evaporation of the organic phase of a lipid solution (Figure 9.2). Rehydration of the
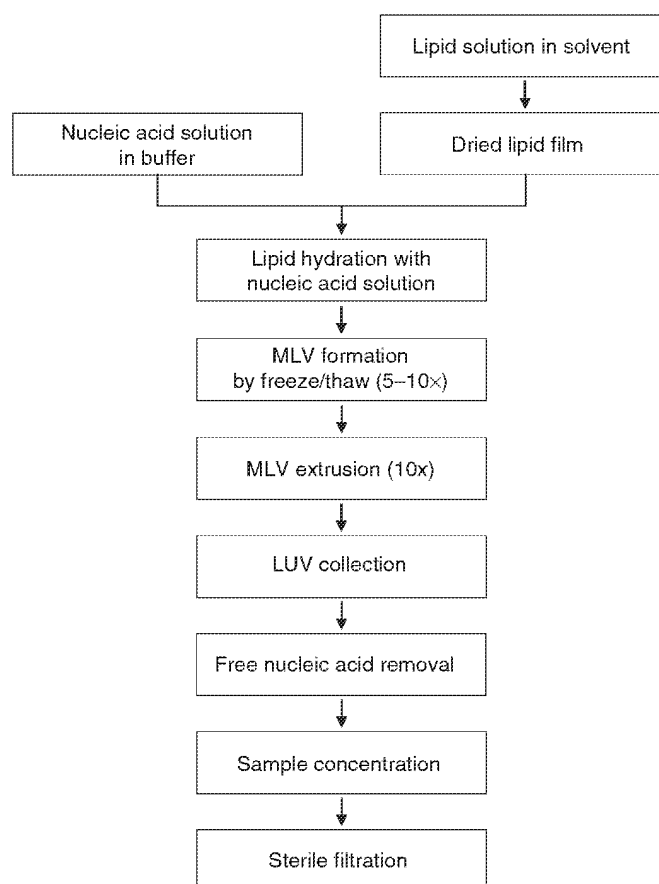
Figure 9.2 Passive method of NA encapsulation. Passive encapsulation utilizes a dried lipid film prepared by evaporating the organic phase of a lipid solution. The resulting lipid film is rehydrated in an aqueous solution of NA in buffer, forming MLV. Multiple freeze-thaw cycles increase the extent of NA encapsulation within the MLV bilayers. The vesicles are then extruded through polycarbonate filters producing LUV.

lipid film in aqueous media, typically buffer containing NA, followed by vigorous mixing, results in the formation of MLV. This is followed by multiple cycles of freezing and thawing to increase the extent to which the NA solute is entrapped by the MLV bilayers. The MLV preparation is then subjected to multiple rounds of extrusion through polycarbonate filters to produce LUV (Figure 9.2 and Figure 9.3) [81]. The size of the LUV is determined by the size of the filter pores. This process suffers from a number of limitations. When used to encapsulate NA, the efficiency of passive encapsulation is generally quite low, ranging from 3 to 45%, depending on the composition of the lipid bilayer and other factors (Table 9.1). The low encapsulation efficiency, consequently, necessitates the incorporation of a postencapsulation separation step such as dialysis, size exclusion chromatography or ultrafiltration to remove nonencapsulated NA. In an effort to improve the efficiency of encapsulation, excess lipid is often incorporated in the formulation process, resulting in low NA/lipid ratios which ultimately impact toxicity and cost of goods. Finally, the extrusion process is inherently difficult to scale. Preparation of large batches requires the use of custom-built extruders to accommodate large filters. The probability of filter tears, resulting in batch failure, increases as the size and cost of the batch increases. In spite of these process limitations, extrusion-based methods for liposome preparation have been successfully adopted by many laboratories, presumably because the technology is readily accessible to the casual investigator. Furthermore, significant progress has been made adapting or enhancing extrusion-based processes for the liposomal formulation of NA-based drugs. These include the use of cationic and anionic lipids [82,83], ionizable cationic lipids [19,84], PEG lipids [85], and detergent or organic solvents such as ethanol [19,60] to control bilayer assembly.
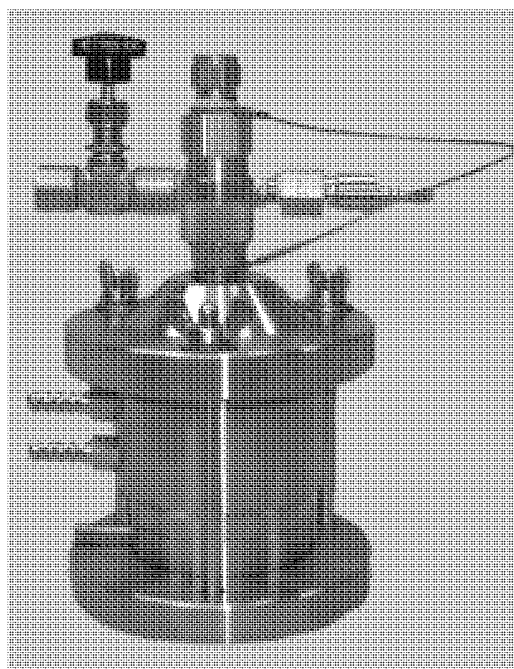
Figure 9.3   The Lipex™ thermobarrel extruder for the preparation of uniformly sized liposomes by extrusion. An MLV or other vesicle preparation is introduced to the top of the extruder and the extruder is pressurized with nitrogen, forcing the MLV through a polycarbonate filter of defined pore size. The resulting LUVs are collected via the outlet port at the bottom of the device. Extrusion is repeated, typically for a total of 10 passes. The unit permits thermostatic operation by virtue of the thermobarrel, which can be coupled to a circulating water bath. Photo courtesy Northern Lipids Inc., Vancouver, Canada, http://www.northernlipids.com.

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Table 9.1 Liposomal Formulations of Oligo- and Polynucleotide Drugs

Formulation Method	Lipid Composition	Size (nm)	Encapsulation (%)	Payload	Reference
1 Passive	DOPE:Chol:Oleic Acid:Palmitoyl-CD4	220±55	<10	ODN	[153]
2 Passive	DPPC:CH:SPDP-PE	~200	3	ODN	[63]
3 Passive	EPC:Chol:DMPG;EPC:DMPG	460±200	<2	ODN	[154]
4 Passive	DOPE:Chol:Oleic Acid	170	10	ODN	[155]
5 Passive	DPPC:Chol:SPDP-PE	100–140	2–3	ODN	[65] #1626
6 Passive	PC:Chol:PS	ND	<10	ODN	[156]
7 Passive	EPC:Chol±Folate-PEG-DSPE	100–140	30–40	ODN	[85]
8 Passive	PC:Chol	110±40	<10	ODN	[157]
9 Passive	DOPE:Chol:Oleic Acid:Palmitoyl-CD4	220±55	<10	ODN	[158]
10 Passive	DDAB:PC:Chol	<2000	>90	ODN	[159]
11 Passive	PC:Chol:PS	ND	<10	ODN	[160]
12 Passive	HVJ liposome: PC:Chol: DC-Chol	ND	60	Ribozyme	[161]
13 Passive	DOGS:DOPE	100–150	88	ODN	[162]
14 Passive	HVJ liposome	ND	ND	ODN	[163]
15 Passive	HVJ liposome:Chol:PC:PS	ND	10–75	Plasmid/ODN	[164]
16 Passive	DSPC:Chol	50–65	ND	RNA Aptamer	[165]
17 Passive	EPC:Chol:Folate-PEG-DSPE	ND	15–20	ODN	[166]
18 Passive	DPPC:Chol:DPPS or DPPA	50–70	24–32	ODN	[92]
19 Passive	HVJ liposome: PS:PC:Chol	ND	ND	pDNA	[167]
20 Passive	HVJ liposome: PC:DOPE: Sph:PS:Chol	ND	ND	ODN	[168]
21 Passive	DPPE:Cetyltrimethyl ammonium bromide	ND	10	ODN	[169]
22 Passive	HVJ liposome	ND	ND	TFD, ODN	[170]
23 Passive	DOPE:CHEMS or SPC	200–300	~16	ODN	[171]
24 Passive	PC40:Chol:PEG-DSPE:DOTAP	<200	80–100	ODN	[93]
25 Passive	EPC:Chol	110+30	10–15	ODN	[172]
26 Passive	HVJ liposome: PS: PC:Chol	ND	ND	ODN	[173]
27 Passive	DPPC:DMPG	316–562	43.5± 4	ODN	[174]
28 Passive	HVJ liposome: PE-DTP:PS:PC:Chol immunoliposomes	400–500	ND	ODN	[175]
29 Passive	CHEMS:DOPE or conventional SPC liposomes	250–300	Up to 20	ODN	[176]
30 Passive	DDAB:EPC:Chol	467.2±72.0	>85	ODN	[177]
31 Passive	HVJ liposome: PS:PC:Chol	ND	ND	ODN	[178]
32 Passive	PE:CHEMS:LLO	240–370	7–15	Various	[179]
33 Passive	Folate liposomes: EPC:Chol:DSPE-PEG-Pteroate	90–110	ND	ODN	[72]
34 Passive	PE:CHEMS:LLO	90–100	10–30	ODN	[180]
35 Passive	Thiocationic lipid: oleic acid:Vitamin D	ND	ND	ODN	[181]
36 Passive	HVJ liposome: PS:PC:Chol	ND	2–5	ODN	[182]
37 Passive	DSPC:Chol:CPL	130	ND	ODN	[183]

(Continued)

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Table 9.1   (Continued)

	Formulation Method	Lipid Composition	Size (nm)	Encapsulation (%)	Payload	Reference
38	Passive	EPC:DPPC:Chol	100	ND	ODN	[184]
39	Passive	DOPC: Tween 20	ND	65	siRNA	[185]
40	Passive	PC:DMPA:Chol	880	ND	ODN, siRNA	[186]
41	Passive	HVJ liposome: EPC:ESM:Chol:DC-Chol	ND	ND	ODN	[187]
42	Passive	EPC:Chol:PEG-PE: DOTAP	50–200	ND	siRNA	[188]
43	Ethanol drop—SALP	PC:Chol:DODAP:PEG-Cer-C_{14} or -C_{20}	110±30	50–80	ODN	[19]
44	Ethanol drop—SALP	DOPE:Chol:DODAC:PEG-Ceramides	100–120	43–57	ODN	[56]
45	Ethanol drop—SALP	DSPC:Chol:DODAP:PEG-Cer-C_{14}	110±30	ND	ODN	[189]
46	Ethanol drop—SALP	DOPE:Chol:DODAC:PEG-C er-C_{14}	100–120	43–57	ODN	[190]
47	Ethanol drop—SALP	DODAP:DSPC:Chol: PEG-Cer-C_{14}	~130	ND	ODN	[191]
48	Ethanol drop—SALP	DODAP:DSPC:Chol:PEG-Cer-C_{14}	~130	ND	ODN	[192]
49	Ethanol drop—SALP	EPC:Chol:DODAP	100–200	57–85	ODN	[193]
50	Ethanol drop—SALP	DC-Chol:EPC:PEG-DSPE	80–90	70–80	ODN	[194]
51	Ethanol drop—SALP	DC-Chol:EPC:PEG-DSPE, Transferrin-PEG-DSPE	100–150	70–80	ODN	[195]
52	Reverse-phase evaporation	"Charge-neutralized liposome"	188	85–95	ODN	[76]
53	Reverse-phase evaporation	HSPC:DSPE-PEG:DOTAP:DSPE-PEG-MAL:Chol	70–120	80–90	ODN	[75]
54	Reverse-phase evaporation	CHEMS:DOPE, CHEMS:DOPE:PEG-PE	ND	ND	ODN	[196]
55	Reverse-phase evaporation	HSPC:Chol:PEG-DSPE	150–190	80–100	ODN	[77]
56	Reverse-phase evaporation	PE:CHEMS:Chol DPPC:DPPG:Chol	>200	10–14	ODN	[197]
57	Reverse-phase evaporation	DODAC:DOPE:PEG-DSPE:PEG:DMPE	<200	>95	ODN	[198]
58	Reverse-phase evaporation	HSPC:DSPE-PEG:DOTAP:Rho-PE: DSPE-PEG-Maleimide	110–130	90	ODN	[199]
59	Reverse-phase evaporation	DOTAP:Chol:HSPC: PEG-DSPE or MAL-PEG-DSPE	100–140	90–95	ODN	[200]
60	Reverse-phase evaporation	DODAP:Chol:PC:PEG-DSPE	150–200	80–100	ODN	[201]
61	Reverse-phase evaporation	DOTAP, POPC,CHOL, MPB-PE, PEG-DSPE	<180	>90	ODN	[202]
62	Ethanol-destabilized liposomes	DSPC:Chol:PEG-Cer-C_{14}:DOTAP	70–120	90	ODN/Plasmid	[60]
63	Ethanol dilution—SNALP	DSPC:Chol:PEG-C-DMA:various cationic lipids	132–182	67–85	siRNA	[20]
64	Ethanol dilution—SNALP	DSPC:Chol:PEG-C-DMA:DLinDMA or DODMA	100–130	90–95	siRNA	[127]
65	Ethanol dilution—SNALP	DSPC:Chol:PEG-C-DMA:DLinDMA	140±12	93±3	siRNA	[14]
66	Ethanol dilution—SNALP	DSPC:Chol:PEG-C-DMA:DLinDMA	100–130	90–95	siRNA	[143]
67	Ethanol dilution—SNALP	DSPC:Chol:PEG-C-DMA:DLinDMA	73–83	92–97	siRNA	[15]
68	Ethanol dilution—SNALP	DSPC:Chol:PEG-C-DMA: DLinDMA	71–84	90–95	siRNA	[61]

9.3.2   The Ethanol Drop (SALP) Method of Nucleic Acid Encapsulation

Stabilized antisense-lipid particles (SALPs) were developed as a means of improving both the limited efficiency of passive NA encapsulation and the pharmacology of the resulting particles. SALPs are prepared by dropwise addition or injection of an ethanolic lipid solution to an aqueous solution of NA, followed by extrusion through polycarbonate filters [19] (Figure 9.4). By utilizing an ionizable aminolipid at an acidic pH, where the aminolipid is fully charged, highly efficient (up to 70%) encapsulation may be achieved. Furthermore, the use of an ionizable lipid facilitates adjustment of the total charge of the system by simply changing the pH after the encapsulation step. In this manner, antisense oligonucleotides may be encapsulated in lipidic systems at NA/lipid ratios as high as 0.25 (w/w) [19]. At the higher NA/lipid ratios novel small multilamellar vesicles (SMLVs) are formed, consisting of numerous (typically 6–9) lamellae arranged concentrically around a dense core. At lower drug to lipid ratios more typical LUVs or capped-LUVs are formed.

9.3.3   Encapsulation of Nucleic Acid in Ethanol-Destabilized Liposomes

An alternative to the SALP method uses ethanol-destabilized cationic liposomes [60,86] (Figure 9.5). This method requires empty liposome formation by extrusion prior to addition of NA. Once cationic liposomes of the desired size have been prepared, they are destabilized by the addition of ethanol to 40% v/v. Destabilization of preformed vesicles requires the controlled addition of ethanol to a rapidly mixing aqueous suspension of vesicles, to avoid formation of localized areas of high ethanol concentration ($> 50\%$ v/v) that promote the fusion and conversion of liposomes into large lipid structures. The addition of NA to ethanol-destabilized liposomes must also be accomplished carefully, in a dropwise manner, to avoid aggregation of the resulting particle suspension. The required extrusion step and the sensitive nature of both the vesicle destabilization
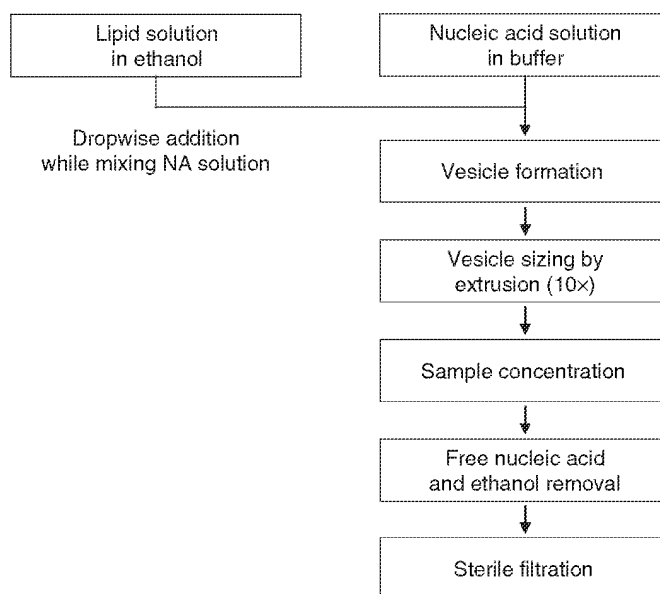
Figure 9.4   Ethanol drop (SALP) method of NA encapsulation. The ethanol drop or SALP method involves the dropwise addition of an ethanolic solution of lipid to an aqueous solution of NA, resulting in the formation of MLV. Vesicles are then sized by extrusion through polycarbonate filters. This method allows for the encapsulation of antisense oligonucleotides with up to 70% efficiency. Either SMLV or LUV can be prepared using this process, depending on the starting NA/lipid ratio.
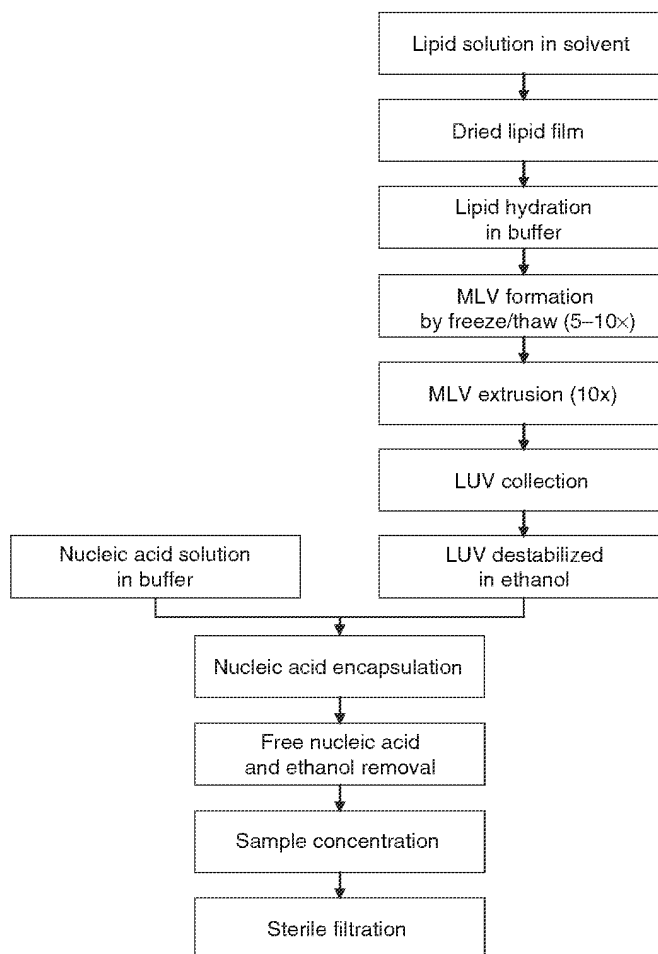
Figure 9.5 Encapsulation of NA in ethanol destablized liposomes. A dried lipid film is rehydrated in buffer, resulting in the formation of MLV. Multiple freeze-thaw cycles follow, and the empty vesicles are then extruded through polycarbonate filters, producing LUV. The LUVs are then destabilized by the controlled addition of ethanol to the rapidly mixing aqueous suspension of vesicles. NA solution is added to the destabilized liposomes in a drop wise manner resulting in encapsulation.

and NA addition represent process challenges that must be overcome prior to adopting this method for the reproducible preparation of encapsulated NA at a scale suitable for clinical evaluation.

9.3.4 The Reverse-Phase Evaporation Method of Nucleic Acid Encapsulation

Reverse-phase evaporation, an effective means of preventing the aggregation of charged liposomes, has previously been used to encapsulate plasmid DNA [87–91] and more recently antisense oligonucleotides [75,92]. The coated cationic liposomes (CCL) developed by Allen et al. utilize a reverse-phase evaporation procedure to accomplish NA encapsulation [75,76,93]. The CCL process is comprised of two stages (Figure 9.6). In the first, hydrophobic cationic lipid–NA seed particles are formed. In the second, the cationic particles are coated with neutral lipids and vesicles are formed by reverse-phase evaporation. The formation of the cationic lipid–NA intermediate is performed by combining two immiscible fluids, an organic solution of cationic lipid in chloroform and an aqueous solution of NA. Addition of methanol results in the generation of a Bligh–Dyer
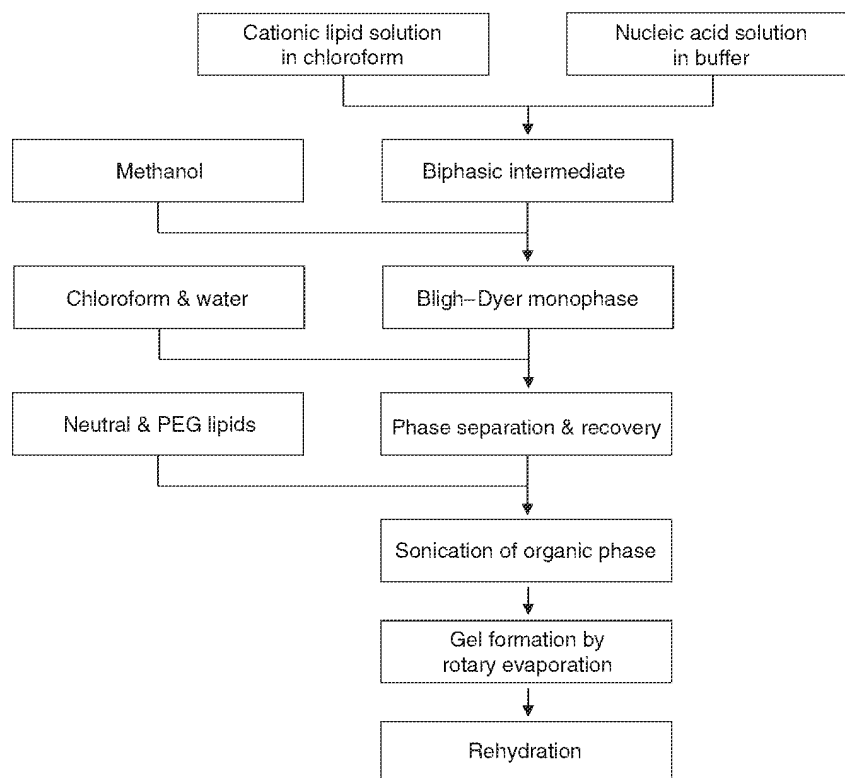
Figure 9.6   Reverse-phase evaporation method of NA encapsulation. The combination of cationic lipid solution in chloroform, and aqueous NA solution in the first step of the reverse-phase evaporation method results in the formation of hydrophobic cationic lipid–NA seed particles. Methanol is added, producing a Bligh–Dyer monophase. Upon reconstitution with excess chloroform and water, the hydrophilic NA is drawn into the organic phase along with the cationic lipid. Neutral lipids are then added, and the organic phase is sonicated and subsequently evaporated to a gel phase. The rehydration step results in NA encapsulated in lipid vesicles ranging from 300 to 600 nm in size.

monophase [94]. When the two-phase system is reconstituted by the addition of excess chloroform and water, the hydrophilic NA is drawn into the organic phase in association with the cationic lipid. Neutral lipids are added and the organic phase is sonicated prior to evaporation to a gel phase. Rehydration results in formation of 300–600 nm vesicles encapsulating NA. Sizing is accomplished via extrusion and unencapsulated NA is removed by size exclusion chromatography.

9.3.5   The Spontaneous Vesicle Formation by Ethanol Dilution (SNALP) Method of Nucleic Acid Encapsulation

The previously described formulation methods rely on the incorporation of an extrusion step to facilitate preparation of small, monodisperse liposomes. The stable nucleic acid lipid particle (SNALP) method was developed specifically as an alternative to these extrusion-based methods [13]. Originally conceived as an alternative to a detergent dialysis method used to encapsulate plasmid DNA, the method has subsequently been adapted to the encapsulation of smaller NA payloads. The detergent dialysis method of plasmid encapsulation involves the simultaneous solubilization of hydrophobic (cationic and helper lipid) and hydrophilic (PEG lipid and plasmid DNA) components in a single detergent-containing phase [55,57]. Particle formation occurs spontaneously upon removal of the detergent by dialysis. This technique results in the formation of small (~100 nm diameter) stabilized plasmid

lipid particles (SPLPs) containing one plasmid per vesicle in combination with optimized plasmid trapping efficiencies approaching 70%.

Although SPLP show considerable potential as systemic gene transfer agents [55,95,96], the detergent dialysis manufacturing method suffers from a number of limitations. Detergent dialysis is exquisitely sensitive to minor changes in the ionic strength of the formulation buffer. Changes as small as 10 mM result in a dramatic decrease in encapsulation efficiency [55,57]. Even when SPLPs are formed under ideal conditions the detergent dialysis method results in the formation of large numbers of empty vesicles that require separation from SPLP by gradient ultracentrifugation. The detergent dialysis process is also difficult to scale to the size required to support preclinical and clinical development of the technology. Finally, detergent dialysis is very inefficient when used to encapsulate smaller NA species such as siRNA duplexes or antisense DNA oligonucleotides. For these reasons, alternative methods of preparing SPLP were explored and a more simple, robust, and fully scalable method for the encapsulation of plasmid DNA has been developed. This method, termed "stepwise ethanol dilution," produces SPLP with the same desirable properties as those prepared by detergent dialysis [13]. Lipid vesicles encapsulating plasmid DNA are formed instantaneously by mixing lipids dissolved in ethanol with an aqueous solution of DNA in a controlled, stepwise manner (Figure 9.7). Combining DNA and lipid flow streams result in rapid dilution of ethanol below the concentration required to support lipid solubility. Using this method, vesicles are prepared with particle sizes <150 nm and DNA encapsulation efficiencies as high as 95%. When the method is adapted to the encapsulation of smaller NA species, vesicle sizes as low as 45 nm are readily obtained and encapsulation efficiencies of 95% are routine. The term SNALP, is used to differentiate from particles prepared using the SALP and SPLP methods, and to denote the more generally applicable methodology which can be applied to any charged NA species.

The ability of the ethanol dilution method to rapidly prepare liposomes of desirable size and encapsulate NA with high efficiency is thought to result from the precise control of the conditions
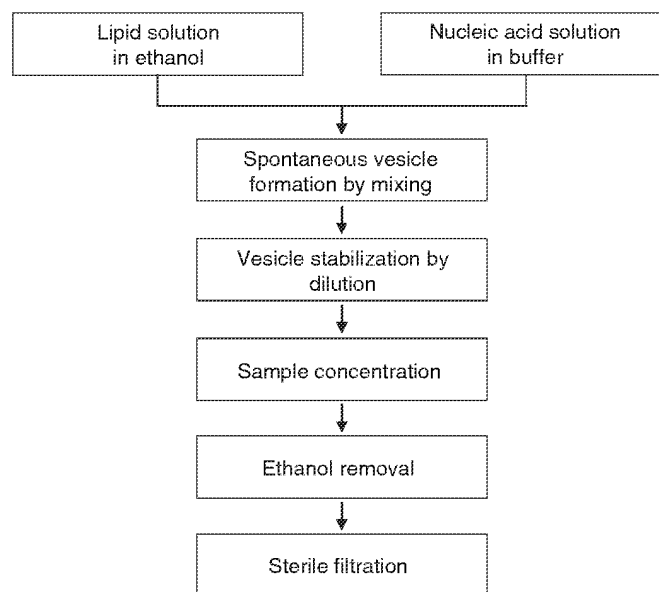
Figure 9.7 Ethanol dilution (SNALP) method of NA encapsulation. The ethanol dilution or SNALP method involves in-line mixing of lipids dissolved in ethanol with nucleic acid dissolved in buffer, resulting in the spontaneous formation of lipid vesicles. As the solutions are mixed, ethanol is diluted below the concentration required to maintain lipid solubility, resulting in vesicle stabilization. Controlled particle sizes from 40 to 150 nm, and encapsulation efficiencies of up to 95% are routinely observed. No extrusion steps are required.

under which the lipids enter the aqueous environment, self-arrange into lipid bilayer fragments, and then form liposomes. By analogy, similar parameters have been shown to be critical for SPLP formation and plasmid encapsulation when using detergent dialysis [95,97]. Ionic strength, cationic lipid, and PEG lipid content must be optimized to maximize plasmid entrapment and minimize aggregation or the formation of empty vesicles [97]. The first stage of dilution is proposed to result in the formation of macromolecular intermediates, possibly lamellar lipid sheets or micelles. NA is recruited to these bilayer fragments by electrostatic attraction. If the cationic lipid content is too low, the plasmid fails to associate with these intermediates, favoring the formation of empty vesicles. If the cationic lipid concentration is too high, the surface charge on the lipid intermediate attracts excess NA, leading to the formation of polydisperse aggregates. At optimal cationic lipid concentrations, NA is proposed to associate with the lipid intermediates in such a way as to reduce the net positive charge on the lipid surface. Association of additional lipid leads to the formation of vesicles containing encapsulated NA. Similar to detergent dialysis, SNALP formation by ethanol dilution is optimized by balancing ionic strength, cationic lipid, and PEG lipid content. However, the ethanol dilution method appears much more robust than detergent dialysis, with good results achieved through a wide range of formulation conditions.

In summary, a variety of techniques are available for encapsulating NA into lipid-based systems. Stepwise ethanol dilution, the SNALP approach, generates small (diameter <100 nm), well-defined, stable systems with high encapsulation efficiencies (>95%) and a broad range of NA/lipid ratios (>0.1 w/w) that exhibit the extended circulation lifetimes required to achieve preferential accumulation at target sites such as solid tumors or liver. Among the various methods for encapsulating NA, stepwise ethanol dilution most adequately satisfies demands related to scalability and reproducibility.

9.4 ANALYTICAL METHODS

An important adjunct to any method of preparing liposomes for NA delivery is the characterization of the resulting system using appropriate analytical methodology. The critical measurements are those that determine the size and monodispersity of the particle preparation, the degree of NA encapsulation, and the particles' surface charge. Since each of these attributes has the potential to affect the pharmacology of a liposomal NA delivery system and each has the potential to change over time, it is critical to develop an understanding of each system's properties and their stability by monitoring each of these parameters using the appropriate methodology.

9.4.1 Measuring Particle Size

Two methods are commonly used to determine the size of a liposome preparation. The first is direct visualization using scanning or transmission electron microscopy. The second is an indirect method, dynamic light scattering, also referred to as quasi-elastic light scattering (QELS) or photon correlation spectroscopy (PCS). Dynamic light scattering measures the size of liposomes suspended in a liquid. A colloidal liposome preparation is in a state of random movement due to Brownian motion. The speed of any given particle is inversely proportional to its size and smaller liposomes move more quickly than their larger counterparts. When a suspension of liposomes is illuminated with a laser, the movement, and therefore the size of the liposomes, can be measured by analyzing the rate at which the light intensity fluctuates as a result of light scatter.

It is important to understand that depending on which method is used to measure the size of a liposome preparation, one can, and will, generate different results. Examination of liposomes under an electron microscope provides a two-dimensional image. Generally, we assume that the ideal liposome is spherical, while in reality, especially on an electron microscope grid, there is infinite number of diameters that can be measured. If the maximum length is used as the diameter, then the

particle is assumed to be a sphere of this maximum dimension. Using the minimum diameter will obviously produce a different result for the particle size.

The situation becomes more complex when we consider the problem of describing a liposome preparation that consists of one or more populations of particles with different sizes. If we imagine a photograph taken with an electron microscope of a liposome preparation consisting of three spheres of diameters 50, 100 and 150 nm, how do we determine and express the average size of the liposomes?

If we simply add all the diameters ($\sum d = 50$ nm $+ 100$ nm $+ 150$ nm) and then divide by the number of liposomes ($n = 3$), the average diameter is 100 nm. This is the mean, or more specifically the number–length mean diameter [98]. The designation "number–length" mean is used, because the number of particles appears in the equation:

$$D[1,0] \qquad \text{Mean diameter} = (50\,\text{nm} + 100\,\text{nm} + 150\,\text{nm})/3 = 100\,\text{nm} = \sum d$$

This value is referred to as $D[1,0]$ because the diameter terms in the numerator are to the power of one (d^1) and there are no diameter terms (d^0) in the denominator of the equation [98]. Manual analysis of photomicrographs yields $D[1,0]$. Automated image analysis of the same photomicrograph would typically begin by measuring the surface area of each liposome to determine the average size. This compares liposomes on the basis of their surface area. Since the surface area of a sphere is $4\pi r^2$, the diameters are squared, divided by the number of particles, and the square root is taken to derive the mean diameter:

$$D[2,0] \qquad = \sqrt{\{(50\,\text{nm}^2 + 100\,\text{nm}^2 + 150\,\text{nm}^2)/3\}} = 108\,\text{nm} = \sqrt{\sum d}$$

This yields the number–surface mean diameter. Since the diameter terms in the numerator are to the power of two (d^2) and there are no diameter terms (d^0) in the denominator of the equation, this value is described as $D[2,0]$ [98]. Our hypothetical example, when analyzed in this way, gives a number–surface mean diameter of 108 nm.

These calculations require explicit knowledge of the absolute number of liposomes analyzed (n), however many instrumental methods determine $D[4,3]$, the volume moment mean, using methods which do not require explicit knowledge of the number of particles analyzed. For example, dynamic light scattering instruments often generate the $D[4,3]$ or the equivalent–volume mean diameter [98].

$$D[4,3] \quad (50\,\text{nm}^4 + 100\,\text{nm}^4 + 150\,\text{nm}^4)/(50\,\text{nm}^3 + 100\,\text{nm}^3 + 150\,\text{nm}^3) = 136\,\text{nm} = \sum d^4 / \sum$$

In this case, the calculated equivalent–volume mean diameter is 136 nm, a difference of 36% relative to the value of $D[1,0]$, the result of manual analysis of data acquired using an electron microscope. These examples, derived from the work of Rawle [98], illustrate how different methods of determining average particle size may yield different results. Often, investigators give extra weight to data acquired by electron microscopy, perhaps because the data acquisition methods seem more direct or "hands on" or because the lower numbers are thought to reflect a higher quality liposome preparation. However, size measurements made using photomicroscopy typically contain $\pm 3 - \pm 5\%$ error. If number–length diameter measurements containing $\pm 4\%$ error are then used to calculate volume mean diameter, a cubic function of the diameter, the error will be cubed upon conversion and will increase to $\pm 64\%$. However, dynamic light scattering can be used to calculate the volume mean diameter with reproducibility approaching $\pm 0.5\%$ [98]. Converting this figure into a number mean gives an error that is the *cube root* of 0.5%.

Furthermore, while electron microscopy allows for the direct examination of liposomes, it is not suitable as an in-process or quality control technique. Sample preparation for electron microscopy is laborious and slow, and a limited number of particles can be examined, increasing the danger of unrepresentative sampling and magnification of error.

9.4.2 Zeta Potential

Zeta potential is a measure of the electric charge acquired by a liposome. This is of interest for two reasons. In the first case, the charge affects particle stability; in the second case the charge affects liposomal pharmacology. Liposomes, as colloidal particles, are subject to the DVLO theory [99,100]. This theory suggests that the stability of a colloidal system is governed by both the repulsive electrical double layer and the attractive van der Waals forces which the particles experience as they approach one another. The energy barrier presented by the repulsive forces must be large enough to prevent particles from contacting one another, adhering and forming aggregates. If this energy barrier is overcome the attractive van der Waals forces will pull the particles into contact and keep them together, an unsatisfactory situation for a liposomal preparation designed to be used as a drug. The goal of liposomal formulation is to prepare a stable, monodisperse particle preparation that retains both monodispersity and particle size in an effort to yield consistent performance. Since charge is a good measure of the magnitude of the interaction between particles, the zeta potential gives an indication of the potential stability of a liposomal system. Liposomes with a large negative or positive zeta potential will repel each other and remain monodisperse and stable. If liposomes have low zeta potential values then the attractive van der Waals forces are able to overcome the repulsive electrical double layer forces, the particles come together, aggregate, and the formulation tends to be unstable. As a rule, liposomes with zeta potentials more positive than $+30$ mV or more negative than -30 mV are considered stable. Particles with low zeta potentials between -30 and $+30$ mV are normally unstable. This would suggest that liposomes should be prepared such that they carry substantial surface charge to enhance their stability as a monodisperse particle preparation. This does not take into account the complex electrostatic milieu encountered once the liposome leaves the vial and enters the blood compartment. Once in the blood, liposomes are free to interact with blood components such as proteins, lipoproteins, and cell surface membranes. Many of these entities are charged and as such, exert either attractive or repulsive forces on the liposomes depending on the charge differential. For this reason, liposomes with substantial positive or negative charge (zeta potential), although stable upon formulation, are rapidly cleared upon systemic administration [101,102]. This presents a dilemma in the design of liposomal systems for the delivery of NA. NA formulations generally incorporate cationic lipids to encourage interaction of the anionic NA with the lipid bilayer. The resulting systems are often highly charged, and accordingly have no appreciable circulation lifetime in systemic applications. In an effort to improve upon the pharmacology of liposomes containing cationic lipids a number of strategies have been adopted including steric stabilization using lipid conjugates of hydrophilic polymers such as PEG. PEG lipids have the undesired side effect of confounding zeta potential readings. For this reason other methods may be necessary for determining the apparent surface charge of PEGylated systems, such as those that utilize fluorescent dyes, for example the toluene nitrosulfonic acid (TNS) assay [20]. The situation is further complicated when using titratable lipids in which case surface charge measurements are specific to the medium in which they are obtained.

9.4.3 Encapsulation

The pharmacology of a liposomal formulation of NA will be largely determined by the extent to which the NA is encapsulated inside the liposome bilayer(s). Encapsulated NA will be protected from nuclease degradation, while those that are merely associated with the surface of a liposome will be less protected. Encapsulated NA shares the extended circulation lifetime and biodistribution

of the intact liposome, while those that are surface associated will adopt the pharmacology of naked NA once they disassociate from the liposome surface. For this reason encapsulation must be accurately determined. An acceptable method is the use of a membrane-impermeable fluorescent dye exclusion assay. This method requires a dye that has enhanced fluorescence when associated with NA. Specific dyes are available for the quantitative determination of plasmid DNA, single-stranded deoxyribonucleotides, and single- or double-stranded ribonucleotides. Encapsulation is determined by adding the dye to a liposomal formulation, measuring the resulting fluorescence and comparing it to the fluorescence observed upon addition of a small amount of nonionic detergent. Detergent-mediated disruption of the liposomal bilayer releases the encapsulated NA, allowing it to interact with the membrane-impermeable dye. NA encapsulation is calculated as $E = (I_o - I)/I_o$, where I and I_o refer to the fluorescence intensities before and after the addition of detergent [55]. Although other methods have been used to determine the liposomal encapsulation of NAs, including nuclease protection assays, chromatographic separation [43], density gradient ultracentrifugation [103], and capillary electrophoresis [104], this method is the most accurate, rapid, and cost-effective. Methods that rely on nuclease protection or chromatographic separation often fail to differentiate encapsulated NA from that which is merely surface associated or trapped in lipid–NA aggregates.

9.5   PHARMACOLOGY OF LIPOSOMAL NA

Systemic delivery to disseminated target tissues requires the use of a "stealthy," relatively charge neutral delivery system, since indiscriminate interaction with blood components, lipoproteins or serum opsonins, can cause aggregation before the carrier reaches the target site. This is especially important in the case of systems containing large polyanionic molecules such as NA, which have a greater potential for inducing toxicity through interaction with complement and coagulation pathways [105]. Other barriers to delivery may include the microcapillary beds of the "first-pass" organs, the lungs and the liver, and the phagocytic cells of the reticuloendothelial system. Accessing target cell population requires the ability to extravasate from the blood compartment to the target site. Charge neutral carriers of appropriate size can pass through the fenestrated epithelium found in sites of clinical interest such as tumors, sites of infection, inflammation, and in the healthy liver and accumulate via the EPR effect [106] (also referred to as "passive" targeting or "disease site" targeting). To take advantage of this EPR effect, which can result in profound enrichment at the target site, carriers must be small (diameter on the order of 100 nm) and long circulating (extended circulation lifetimes following intravenous injection in mice). Clearly, NA stands to benefit from the pharmaceutical enablement conferred by encapsulation in appropriately designed liposomal carriers.

9.5.1   Pharmacokinetics and Biodistribution of Liposomal NA Following Systemic Administration

Following intravenous injection, the clearance of properties of encapsulated NA can be assessed by lipid and/or NA markers. (As methods of determining the pharmacokinetics and biodistribution of NA themselves are described elsewhere in this volume they will not be discussed here.) Previous experience shows that, if NA is fully encapsulated in stable liposomes, the lipid and NA components are cleared from the blood compartment at the same rate and the NA remains intact, protected from nuclease degradation while encapsulated within the liposome [15,107, **AQ1** 14]. As long as the liposome remains intact, the biodistribution of a nonexchangeable lipid marker [108] incorporated in the formulation is representative of the biodistribution of the entire particle, including the NA component. This finding may be applied to analysis of liposomal clearance and biodistribution up to 24 h after administration, after which time even the most stable lipid markers will begin to experience some remodeling or exchange [109].

LIPOSOMAL FORMULATIONS FOR NUCLEIC ACID DELIVERY   **255**

A comparison of the clearance properties of liposomal formulations of siRNA in three different species is shown in Figure 9.8. Liposomes were formulated containing DSPC, cholesterol, DLinDMA [AQ2] and PEG-c-DMA encapsulating siRNA. The specific liposome composition, manufactured using the SNALP process, was selected for efficient delivery to the liver, with a view towards avoiding accumulation in distal tissue or in nontarget tissues of the reticuloendothelial system such as the spleen. The dose remaining in plasma and tissue samples obtained at various times after intravenous administration in either mice or guinea pigs was determined using the radiolabeled lipid marker ([^{3}H]-cholesteryl hexadecyl ether [CHE]) [15,61]. The plasma clearance properties of liposomally encapsulated siRNA in cynomolgus monkeys was determined directly by ion exchange high-performance liquid chromatography (HPLC) [15]. Four hours after tail vein injection in mice, $3.3 \pm 1.3\%$ of the injected dose remains in the plasma with a half-life of 38 min. The half-life of unprotected, unmodified phospodiester siRNA has been shown to be < 2 min in mice [14]. When liposomal siRNA is administered intravenously via ear vein injection in guinea pigs, $3.0 \pm 1.0\%$ of the injected dose remains in the plasma 4 h after administration, corresponding to a plasma half-life of 39.3 min [61]. When encapsulated siRNA is administered to cynomolgus monkeys as a bolus injection in the saphenous vein, 17% of the injected dose remains in the plasma after 4 h, corresponding to a plasma half-life of 72 min. The agreement between the clearance properties in mice and guinea pigs, especially given the different routes of administration, is remarkable. Also noteworthy is the extent to which the doubling in the plasma half-life as measured in mice and primate species is predicted based on the comparative pharmacologic studies which have given rise to the technique of allometric scaling, whereby the pharmacological parameters of a given drug can be predicted in different species [110].

Using either radiolabeled lipid markers or direct analysis of NA, the biodistribution of liposomal NA following intravenous administration may be determined. Figure 9.9 illustrates the accumulation of liposomal siRNA in various tissues 24 h after the administration in mice and guinea pigs. The liver and spleen typically demonstrate the highest levels of liposome accumulation. In this case the liver has accumulated 70.7 ± 5.4 and $83.4 \pm 6.5\%$ of the injected dose per gram, in mice and guinea pigs, respectively and the spleen has accumulated 0.94 ± 0.15 and $2.2 \pm 0.2\%$ of the injected dose per gram in mice and guinea pigs, respectively; whereas, the kidney, heart and brain accumulate the least amount of liposomal NA. Of note, the kidney, the prototypical target tissue associated with the toxicity of naked antisense drugs, accumulates <1% of the injected dose per gram, in both mice and guinea pigs.
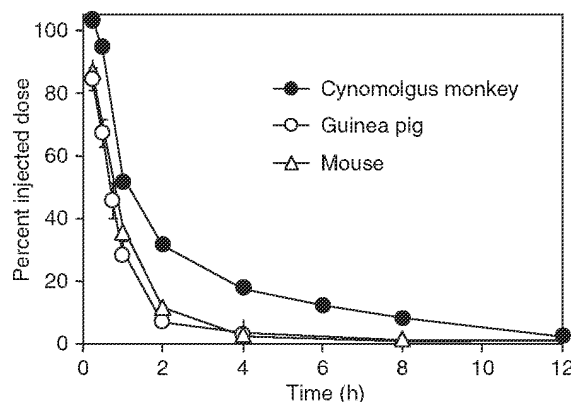
Figure 9.8   Plasma clearance of liposomal (SNALP) encapsulated siRNA. Plasma clearance of SNALP siRNA determined in mice, guinea pigs, and cynomolgus monkeys. Each animal received a single intravenous injection of SNALP-formulated siRNA. Data represent percent of the total injected dose in blood at the indicated time points after treatment. Mouse and guinea pig data are presented as mean $\pm$ s.d., $n = 5$. Cynomolgus monkey data represent the mean of two treated animals.
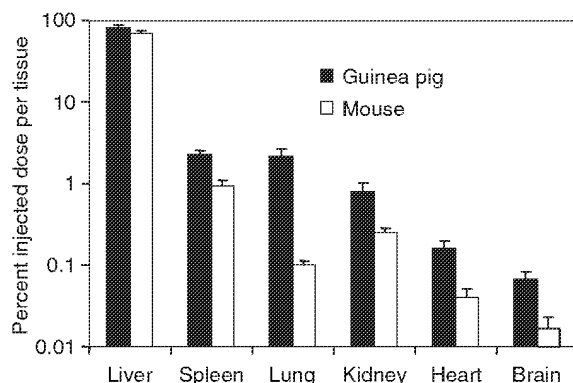
Figure 9.9   Biodistribution of liposomal (SNALP) encapsulated siRNA. Biodistribution of SNALP siRNA was determined in mice and guinea pigs. Each animal received a single intravenous injection of ^{3}H-labeled SNALP-formulated siRNA. Data represent percent of the total injected dose in each tissue 24 h after treatment. Data are represented as mean $\pm$ s.d., $n = 5$.

While these results are typical of freely circulating liposomal systems, the extent to which liposomes accumulate in certain tissues, especially the liver, spleen, and distal disease sites such as tumors, can be modulated by affecting changes in the liposome formulation. Manipulation of the chemistry of the individual lipid components and their relative molar ratios within the system can significantly alter a formulation's pharmacokinetics, biodistribution, and transfection efficiency. One such example of this plasticity is illustrated in Figure 9.10 and Figure 9.11. The plasma clearance and liver accumulation of three liposomal siRNA formulations (SNALP) that differ *only* in the alkyl chain length of the incorporated PEG–lipid are shown. Shorter PEG–lipid anchor lengths decrease the blood circulation times of the SNALP and increase the rate and extent of nanoparticle accumulation in the liver of mice. SNALP containing PEG lipids with distearyl (C18), dipalmityl (C16), and dimyristyl (C14) lipid anchors have circulation half-lives of 4 h, 2 h, and 40 min, respectively. In this example, up to 75% of the total injected dose of PEG-cDMA-containing particles accumulates in the liver after intravenous administration, while 35% of the dose accumulates in the liver when the more stably integrated PEG-cDSA is used. Further manipulation of the liposomal bilayer composition can result in <20% of the total injected dose accumulating in the liver, with concomitant increases in the extent of accumulation in non-RES tissues such as disseminated tumors [58].

The extent of NA distribution in tissues following administration of liposomal systems is markedly greater than what has been observed in other systems. This can be attributed to the extended blood circulation lifetimes of liposomal formulations and their ability to protect encapsulated NA from degradation, greatly extending the available timeframe for delivery to and accumulation within tissues. While liposomal formulations may provide plasma half-lives for intact NA of 0.5–60 h [61, 14, 114, 107, 15   AQ3
] "naked" NA and cationic lipoplex typically have half-lives of minutes
or less [14,111–114].

9.5.2   Toxicity of Liposomal NA Formulations

The raison d'être of drug delivery technology is to improve a drug's effectiveness by increasing availability of the drug at the intended target site. However, an unintended by-product that often accompanies the use of drug delivery technology is a shift in drug-associated toxicity. In many cases these drug-related toxicities may be anticipated by previous experience with the free drug in that the mechanism of toxicity is conserved; however, a shift in the target organ of toxicity is common [115,116].
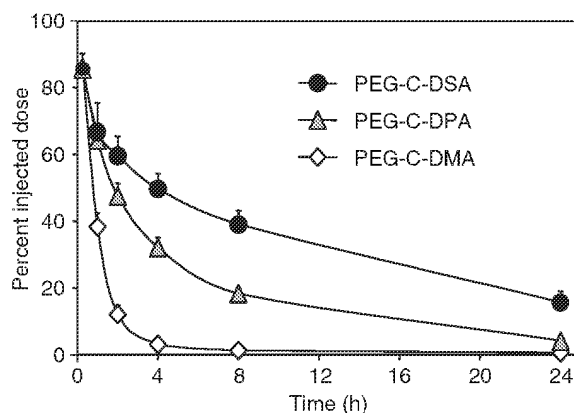
Figure 9.10 Plasma clearance of SNALP containing PEG–lipids with increasing alkyl chain lengths. Plasma clearance of ^{3}H-labeled SNALP containing PEG-C-DMA, PEG-C-DPA or PEG-C-DSA in ICR mice. SNALP were administered i.v. at 5 mg/kg siRNA. Data represent percent of the total injected dose in blood at the indicated time points after treatment. Values are mean ± s.d., $n = 4$ mice.
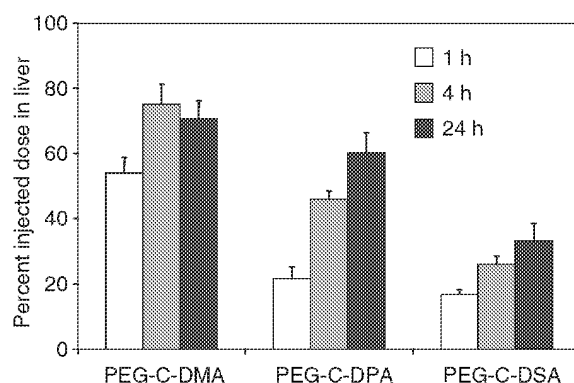
Figure 9.11 Liver accumulation of SNALP containing PEG–lipids with increasing alkyl chain lengths. Liver accumulation of ^{3}H-labeled SNALP containing PEG-C-DMA, PEG-C-DPA or PEG-C-DSA in ICR mice. SNALP were administered i.v. at 5 mg/kg siRNA. Data represent percent of the total injected dose in liver at the indicated time points after treatment. Values are mean ± SD, $n = 4$ mice.

While many small molecule chemotherapeutic drugs in their free form give rise to nephrotoxicity or hepatotoxicity as a result of accumulation in the kidneys or liver, the same drugs, once encapsulated and delivered in liposomal form, may give rise to previously unobserved mucocutaneous toxicities or peripheral neuropathy [115,116]. These changes in toxicity are similar to those observed when using liposomal NA drugs. When naked antisense phosphorothioate oligonucleotides are injected intravenously in mammals, ~20% of the injected dose accumulates in the kidney [117]. At high doses in monkeys this level of accumulation manifests as toxicity, first in the form of focal tubular regeneration, and at even higher doses as perturbation in N-acetylglucosamine, total protein, and retinol-binding protein levels [118]. Similarly, high levels of accumulation in the liver lead to hypertrophy of Kupffer cells and ultimately increases in transaminases such as AST and ALT [119]. Since liposomal encapsulation results in a dramatic shift in the biodistribution of NA we would expect a concomitant shift in the target organs of toxicity. Indeed, as intravenous administration of liposomal NA results in accumulation of <1% of the total injected dose in the kidney when measured in either mice or guinea pigs [61], little or no nephrotoxicity results, even at doses greater than those required to elicit hepatic toxicity as measured by elevations of serum transaminases [15].

A special consideration when working with liposomal systems is their potential to activate the complement system. In particular, liposomes possessing cationic or anionic lipids are capable of binding complement proteins and triggering the activation of the complement cascade [105]. To test liposomal systems for their ability to activate complement, standard in vitro assays may be performed, or complementary experiments may be performed in vivo. Results of numerous studies indicate that complement activation by liposomal NA may be prevented by controlling the amount and presentation of liposomal surface charge, either through the use of PEG–lipids or by adjusting the amount or type of cationic lipid used. PEGylated systems containing modest amounts of titratable cationic lipid appear to be particularly stealthy in this regard [120].

9.5.3   Immune Stimulation

NA can cause activation of the mammalian innate immune system leading to the release of interferons and proinflammatory cytokines. In the case of DNA, immune stimulation is triggered primarily by the recognition of unmethylated CpG sequence motifs by Toll-like Receptor-9 (TLR9) [121] located within the endosomal compartment of certain antigen presenting cells (APC) [122,123]. Similar immune recognition pathways are also activated by exogenous single [124,125] and double-stranded RNA [126] through TLR7/8 and TLR3, respectively. It has been recently reported that synthetic siRNA can also induce potent immune stimulation [127–129]. The immune responses elicited by NAs are greatly potentiated by the use of delivery vehicles, including either liposomal- or polycation-based vehicles that facilitate intracellular delivery via endosomes, the primary intracellular location of the affected TLRs [127,130].

Although the immunomodulatory effects of CpG DNA have potential therapeutic utility in oncology and allergy applications [131], in many other applications immune activation represents an additional hurdle to drug development. The consequences of an unmanaged activation of the innate immune response can be severe, particularly in more sensitive species including humans [132–134] due to the multitude of local and systemic inflammatory reactions that can be triggered by activation. Many of the toxicities associated with the administration of siRNA in vivo have been attributed to this response [14,127].

On the basis of the finding that immune activation by siRNA is sequence-dependent, it is possible to design active siRNA with negligible immunostimulatory activity by selecting sequences that lack GU-rich or other immunostimulatory motifs [127,135]. Although this strategy has proven successful, it significantly limits the number of novel siRNA sequences that can be designed against a given target. Furthermore, it currently requires some degree of screening due to the relatively ill-defined nature of putative RNA immunostimulatory motifs. Another approach involves the use of stabilization chemistries that were previously developed for ribozymes or antisense oligonucleotide drugs [136] that have more recently been applied to the chemical stabilization of synthetic siRNA. siRNA may be designed containing 2′OMe [137–139], 2′F [137,139–141], 2-deoxy [140] or "locked nucleic acid" (LNA) [128,142] modifications yet retaining functional RNAi activity, indicating that these chemistries can be compatible with the RNAi machinery. However, modification of siRNA appears to be tolerated only in certain positional or sequence-related contexts, and in most cases, indiscriminate modification has a negative impact on RNAi activity [128,138–140,142]. Until recently, the design of chemically modified siRNA has required laborious screening in an effort to identify duplexes that retain potent gene silencing activity.

Recently, a more rational approach to the design of highly active, nonstimulatory siRNA molecules has been described [143]. Surprisingly, minimal 2′OMe modifications within one strand of a double-stranded siRNA duplex are sufficient to fully abrogate the immunostimulatory activity of siRNA, irrespective of sequence. Remarkably, incorporation of as few as two 2′OMe guanosine or uridine residues in highly immunostimulatory siRNA molecules completely abrogate siRNA-mediated interferon and inflammatory cytokine induction in human PBMC and in mice in vivo. This degree of chemical modification represents ~5% of the native 2′-OH

positions in the siRNA duplex. Since complete abrogation of the immune response requires only one of the RNA strands to be selectively modified, 2′OMe modifications can be restricted to the sense strand of the duplex, therefore minimizing the potential for attenuating the potency of siRNA, which is predominantly determined by the antisense "guide" strand. Minimally modified siRNA retains potent gene silencing in vivo, without evidence of cytokine induction, immunotoxicities or off-target effects associated with immune activation triggered by unmodified siRNA. This provides a simple method of designing nonimmunostimulatory siRNA based on native sequences with proven RNAi activity.

It is presently unclear how the introduction of 2′OMe nucleotides into one strand of an siRNA duplex prevents recognition of siRNA by the immune system. The trans-inhibitory effect of 2′O-methylation, whereby 2′OMe-modified ssRNA annealed to unmodified immunostimulatory ssRNA generates a nonimmunostimulatory duplex, is consistent with a hypothesis that involves recognition of the siRNA by its putative immune receptor, thought to be TLR-7 [128], as a double-stranded molecule. It is conceivable that 2′OMe-modified siRNA may avoid recognition by the innate immune system using a mechanism that has evolved to allow for the differentiation of self from pathogen-derived RNA.

Other NA modification chemistries have the potential to influence immune stimulation. LNAs containing a 2′-O, 4′-C methylene bridge in the sugar ring have been shown to partially reduce the immunostimulatory activity of siRNA [128]. However, siRNAs containing inverted deoxy abasic end caps retain immunostimulatory activity [14]. No evidence of a trans-inhibitory effect was observed with LNA-modified duplexes. These observations suggest that, for reasons we do not currently understand, immune stimulation by siRNA may be particularly sensitive to inhibition by 2′OMe modifications versus other stabilization chemistries. Minimal 2′OMe modification to prevent the induction of interferons and inflammatory cytokines has been shown to both limit the potential for nonspecific effects on gene expression and improve the tolerability of siRNA formulations. Intravenous administration of liposomal 2′OMe-modified siRNA is efficacious and well tolerated in mice [143]. This approach, coupled with ongoing improvements in AQ4 delivery technology and siRNA design, may be an important component in the development of synthetic siRNA therapeutics.

9.5.4 Immunogenicity

The potential for a drug to be immunogenic is a serious concern in drug development since the establishment of an antibody (Ab) response can severely compromise both the safety and efficacy of a drug. This has hampered the development of drugs, including protein-based therapeutics such as monoclonal antibodies that contain immunogenic components. It has long been recognized that liposomes can act as immunological adjuvants as a result of their particulate nature, efficient uptake by APC, and ability to cross-link surface receptors [144]. This property is enhanced when immunostimulatory agents such as CpG DNA are incorporated into the liposomes [145,146]. This has been exploited in the design of liposomal vaccines that generate strong Ab responses against weakly immunogenic antigens attached to the liposome surface. It is therefore unsurprising that immunogenicity has proven to be a major obstacle in developing receptor-targeted liposomes that incorporate antibodies, peptides or receptor ligands on their surface to enhance target cell uptake [147–149]. Remarkably, the addition of a PEG coating to these liposomes typically has a minor effect on reducing their immunogenicity [145,147,149].

Experience with stable plasmid lipid particles (SPLPs), a liposomal system for the delivery and expression of therapeutic pDNA [13,58], provides an example of the challenges faced when designing nonimmunogenic NA carriers. The in vivo safety and efficacy of SPLP that contain stably integrated PEG lipids are severely compromised following repeat administration due to a surprisingly robust Ab response against PEG that arises from a single administration. The immunogenicity of PEGylated liposomes containing pDNA can be greatly reduced by using alternative diffusible

PEG–lipids that diffuse more readily from the lipid bilayer upon administration. By eliminating the Ab response to PEG, these modified liposomes can be safely readministered to mice while maintaining the effective delivery of the pDNA payload to distal tumor sites. Administration of non-immunogenic SPLP is still associated with substantial cytokine induction, indicating that the reduced immunogenicity is not due to abrogation of the immunostimulatory activity of the pDNA payload. Instead, this supports the hypothesis that robust Ab responses to PEG require the close physical association of the PEG–lipid with pDNA and are driven by the specific binding and internalization of PEGylated liposomes containing stimulatory pDNA by PEG-reactive B cells [120]. An alternative approach to reducing carrier immunogenicity may be the development of less immunostimulatory NAs. CpG-free pDNA and chemically modified antisense ODN or synthetic siRNA may have significantly reduced capacity to activate cytokine responses. Synthetic siRNAs can induce potent immune stimulation in vivo, driving the production of a strong anti-PEG Ab response when immunostimulatory siRNAs are encapsulated in PEGylated liposomes containing stably integrated C18-PEG–lipids [120]. Minimally modified siRNA duplexes, when encapsulated in PEGylated liposomes containing stably integrated PEG–lipids, are nonimmunogenic. Use of minimally modified, nonimmunostimulatory NA and/or diffusible PEG–lipids allows for flexibility in the design of nonimmunogenic liposomal systems.

These findings raise important concerns regarding the potential immunogenicity of any delivery vehicle currently under consideration for use with immunostimulatory NA-based drugs. Given that most RNA and DNA species stimulate innate cytokine responses and B cell activation [124–128,143], vehicle immunogenicity may prove to be problematic for a range of NA-based therapeutics. Antibody responses against surface components, especially targeting ligands, of liposomal systems should be closely monitored. However, the ability to abrogate the immunogenicity of liposomal NA formulations by simple modification of either their lipid composition or NA provides multiple paths forward in the design and clinical development of these systems.

9.5.5   The Efficacy of Liposomally Formulated NA Drugs

Currently, the clinical experience with liposomal formulations of NA drugs is limited, requiring us to rely on preclinical results to gauge their promise. In this regard there are many reports of efficacy associated with liposomal formulations of antisense ODN, ribozymes and more recently siRNA (recently reviewed by Behlke [150]). Liposomal NAs have been evaluated in preclinical models of infectious disease, inflammation, cancer, and various metabolic conditions. However, it is only recently that we have come to appreciate the extent to which nonspecific effects, such as stimulation of the innate immune system, may effect the results obtained in preclinical models that are used to measure efficacy. Particularly troublesome is the impact that induction of the innate immune system has on anti-tumor efficacy in murine models and on models of infectious disease. For these reasons it is especially crucial to adopt appropriate controls when working with these systems. Specifically, the inclusion of nontargeting control NA, with similar immunostimulatory properties to the active compounds, is required. As described in the previous section, with our improved understanding of the chemical mod-ification strategies that abrogate the immune stimulation associated with siRNA, it is now straightfor-ward to design immunologically silent NA that retain their desired mechanism of action [143].

In spite of the well-documented impact that liposomal NA can have on the innate immune system some investigators have failed to fully characterize the immunostimulatory properties of their test article prior to publishing the efficacy results. Others have unwittingly reported false-negative immune stimulation data obtained by analyzing the immunostimulatory properties of their compounds either in cell lines that are not competent for an innate immune response, or by harvesting preclinical samples at inappropriate time points, days or even weeks after the immune stimulus has been applied. It is noteworthy that in our laboratory we have undertaken a retro-spective analysis of published siRNA used in efficacy studies and with one single exception all were shown to be immunostimulatory. All of the siRNA had either been previously described

as nonimmunostimulatory, or their immunostimulatory properties had not been described. Even more alarming is the fact that the one exception in our analysis was a negative control siRNA that has been used in more than 12 published studies to support the "efficacy" of an assortment of highly immunostimulatory siRNA. Control siRNA with similar immunostimulatory properties to the active compounds would have been preferred. Although the various pathways that may be affected by NA drugs are complex, assaying for interferon alpha and IL-6, either 2–12 h after intravenous administration in mice or 24 h after exposure of primary PBMCs, is all that is required to adequately gauge the immunostimulatory properties of most molecules.

When efficacy studies are approached and interpreted with appropriate caution, the results can be substantially more convincing. Rather than provide a retrospective analysis of previously described efficacy studies, here we will describe one example that hopefully illustrates the potential of liposomal formulations of NA drugs, the example of liposomal siRNA targeting apolipoprotein B (apoB). ApoB is an essential component involved in the assembly and secretion of very low-density lipoprotein (VLDL), a precursor to LDL. ApoB is considered "non-druggable" with conventional small molecule therapies yet it is a highly relevant, genetically and clinically validated disease target. Targeting apoB with second-generation antisense oligonucleotides has shown promising preclinical and clinical results [151] and cholesterol-conjugated siRNAs directed against apoB have successfully resulted in knockdown of apoB message yielding a concomitant reduction in total cholesterol [152]. Unlike many oncology, inflammatory or infectious disease targets, the apoB transcript is regulated mainly at the posttranslational level. This is believed to confer some protection from off-target effects that would otherwise result in unintended perturbations in apoB expression levels, making apoB a "good" target for proof of concept efficacy studies.

Intravenous administration of high doses, >50 mg/kg, of apoB-specific siRNA, siApoB-1, as naked siRNA, in the absence of chemical conjugation, has previously shown to have no in vivo silencing activity in mice [152]. However, profound silencing of liver apoB mRNA and apoB-100 protein is achieved with a single, low dose of liposomal (SNALP-formulated) siApoB-1 [15]. No detectable reduction in apoB is observed upon treatment with SNALP-formulated mismatch siRNA (siApoB-MM) or empty SNALP vesicles, indicating that silencing is specific to the siRNA and is not an artifact caused by the liposomal carrier or due to other off-target effects.
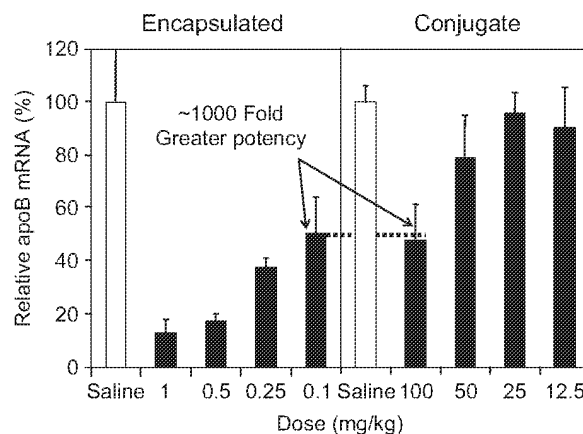
Figure 9.12 The efficacy of liposomal (SNALP) encapsulated siRNA compares favorably to chemically conjugated siRNA. The dose-dependent silencing of liver apoB mRNA after administration of either SNALP siApoB-1 (left panel) or Chol-siApoB-1 (right panel) is shown. Liver apoB mRNA levels were quantified relative to GAPDH mRNA 3 days after i.v. administration of siRNA. Data are mean values relative to the saline treatment group ±s.d. Chol-siApoB-1 was administered at doses of 100, 50, 25 or 12.5 mg/kg (n = 6 per group), and SNALP siApoB-1 was administered at siRNA doses of 1, 0.5, 0.25, and 0.1 mg/kg (n = 4 per group).

Figure 9.12 is an illustration of the relative potency of siApoB-1 SNALP-mediated silencing compared to cholesterol-conjugated siApoB-1. While a dose of 100 mg/kg of cholesterol-conjugated siRNA is required to achieve 50% knockdown of apoB message, comparable levels of gene silencing are achieved at a dose of 0.1 mg/kg SNALP siApoB-1, corresponding to a 1000-fold increase in the potency of siRNA, when it is encapsulated relative to the cholesterol conjugate. This represents an increase in potency relative to the naked, unconjugated molecule that is more than four orders of magnitude. This degree of silencing is readily achieved in the absence of immune stimulation or other toxicities. A nonhuman primate study, using a considerably less potent siRNA sequence, confirmed that liposomal siRNA can potently silence apoB [15]. Silencing of 90% of the endogenous apoB message was achieved in cynomolgus monkeys treated with a single intravenous administration of 2.5 mg/kg SNALP-formulated siApoB-1. Again, this result was achieved in the absence of any toxicity as measured by general tolerability, complement activation, coagulation or proinflammatory cytokine production. There were no changes in hematology parameters for SNALP-treated animals. The only measurable change in SNALP siApoB-2-treated primates was a moderate, transient increase in liver enzymes in monkeys that received the highest dose of SNALP siApoB-2. This manifested as transient transaminosis that peaked at 48-h posttreatment and was highly variable among individual animals. This effect was completely reversible, normalizing within 6 days, while the reduction in apoB had yet to reach its nadir. It is important to consider that a 90% reduction in apoB levels is unlikely to be a relevant clinical target, meaning that lower doses would be used in a clinical context. In monkeys, a more moderate treatment with a dose of 1.0 mg/kg resulted in a 68% in apoB message and a 50% reduction in plasma LDL in the absence of any transaminosis. Subsequent examination of this type of toxicity in mice has revealed a number of opportunities for improving the therapeutic window of apoB SNALP, including the use of more potent siRNA sequences, formulation refinements, and changes to the dosing regime.

While further optimization of NA payloads, formulations, and treatment regimens may be required, the experience with apoB SNALP suggests that effective systemic delivery of NA using liposomes is readily achievable. Together with efforts to develop chemically modified NA with optimal pharmacologic properties, liposomal NA shows considerable promise in a number of applications. It is highly likely that as NA drugs continue to move from bench to bedside, liposomes will increasingly become a component of their success.

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JOINT APPENDIX 32

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United States Patent [19]

Unger et al.

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[45] **Date of Patent:** **Nov. 3, 1998**

[54] **CATIONIC LIPIDS AND THE USE THEREOF**

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[21] Appl. No.: **391,938**

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[52] **U.S. Cl.** **424/1.21**; 424/283.1; 424/450; 436/71; 436/829; 530/300; 536/23.1

[58] **Field of Search** 424/283.1, 450, 424/1.21; 435/71; 436/71, 829; 536/23.1; 530/300

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[57] **ABSTRACT**

Cationic lipid compounds which comprise at least two cationic groups. The cationic lipid compounds are particularly suitable for use as carriers in the intracellular delivery of bioactive agents, including pharmaceuticals and genetic material. Compositions of the present cationic lipid compounds include suspensions, emulsions, micelles and liposomes.

143 Claims, 2 Drawing Sheets

CAT EXPRESSION (ng/ml)
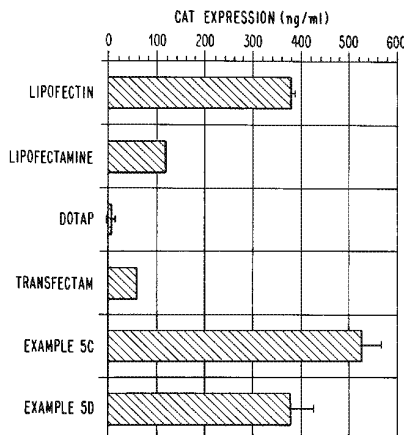
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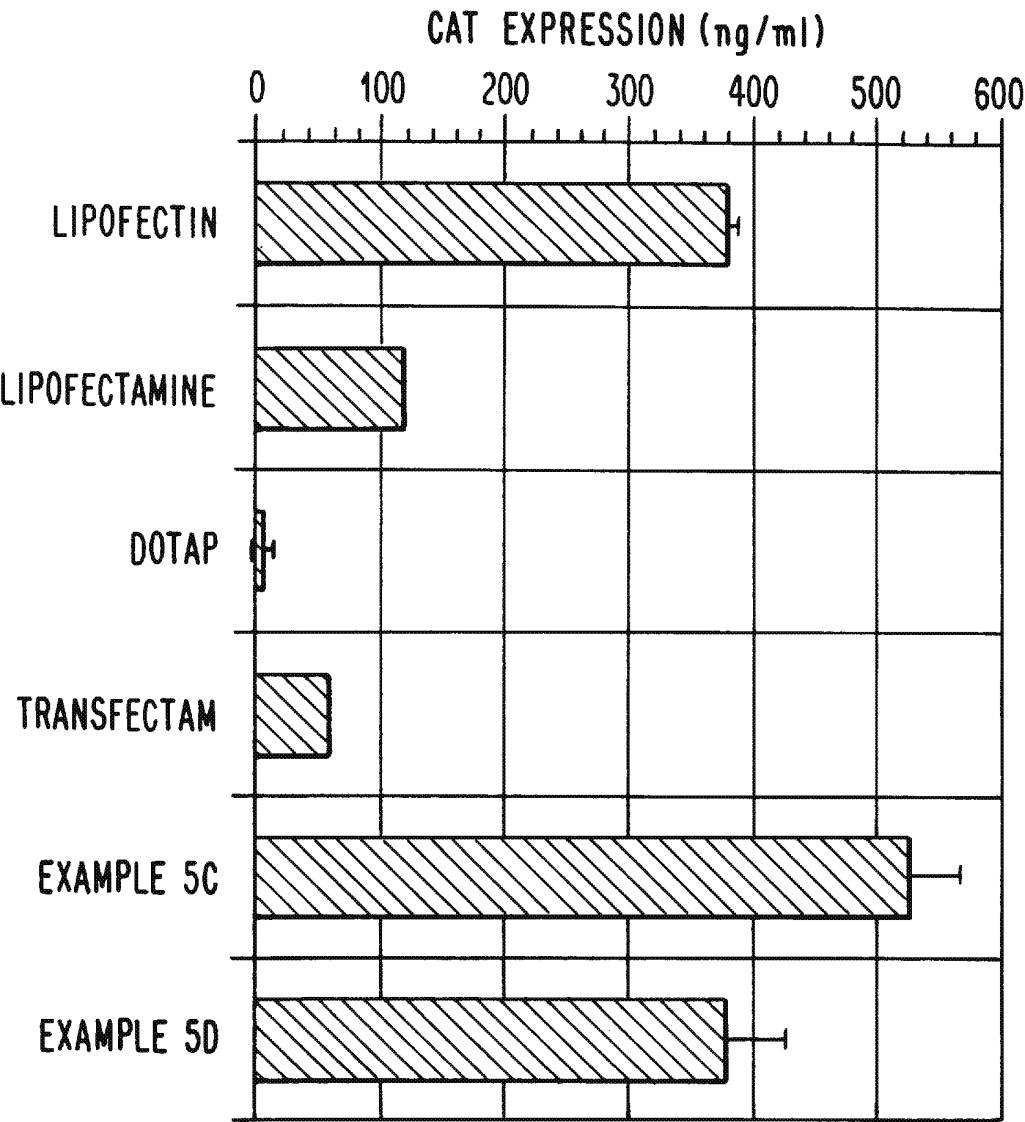
Fig.1
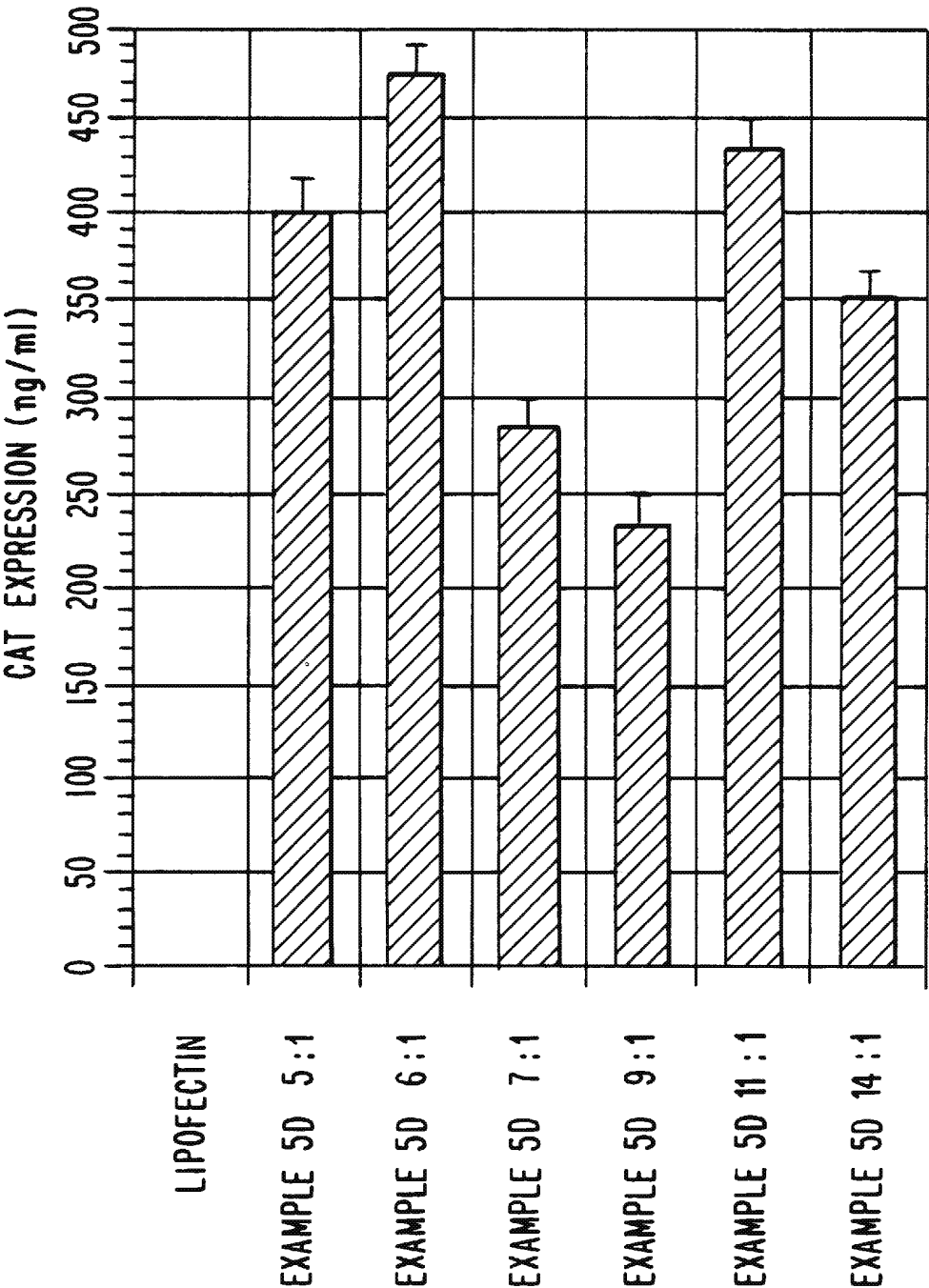
Fig. 2

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CATIONIC LIPIDS AND THE USE THEREOF

FIELD OF THE INVENTION

The present invention relates to novel cationic lipids and the use thereof. More particularly, the present invention relates to novel cationic lipids and their use in the delivery of biologically active agents.

BACKGROUND OF THE INVENTION

The intracellular delivery of biologically active agents, for example, pharmacologically active materials and diagnostic agents, is generally desirable in connection with the treatment and/or diagnosis of various diseases. For example, cell function can be influenced at the subcellular or molecular level by delivering the biologically active agent intracellularly.

Various methods have been developed for the delivery of biologically active agents directly into living cells. Included among such methods is the "carrier method" which involves the use of a carrier to promote intracellular delivery of a bioactive agent to specifically targeted cells, for example, diseased cells. The intracellular delivery of therapeutic agents is referred to herein also as "transfection".

Various carriers have been developed for use in the transfection of biologically active agents. For example, liposomes and polymers have been developed for the transfection of genetic materials, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). However, the currently available carriers, including liposomes and polymers, are generally ineffective for the intracellular delivery of biologically active materials in vivo. Moreover, the currently available carriers have limited use in connection with the transfection of cells in vitro.

In addition to the carrier method, alternative methods have been developed for the transfection of biologically active agents, including genetic material, directly into cells. These methods include, for example, calcium phosphate precipitation and electroporation. However, these methods are also generally ineffective for the intracellular delivery of biologically active agents in vivo.

Great strides have been made in connection with the characterization and understanding of various diseases, for example, genetic diseases, and their associated protein transcription, in humans and other animals. This has led to the development or postulation of improved methods for the treatment of such diseases with biologically active agents. Various of these methods involve or require that the biologically active agent be delivered intracellularly. As noted above, however, current methods for the transfection of cells with biologically active agents in vivo are generally ineffective. This is thwarting the study and implementation of improved methods for the treatment of various diseases.

The cellular membrane is a selective barrier which prevents random introduction of substances into the cell. Accordingly, a major difficulty in the intracellular delivery of biologically active agents is believed to involve the transfer of the agent from the extracellular space to the intracellular space. Localization of the biologically active agent at the surface of selected cell membranes has been difficult to achieve also.

Carriers have been engineered also from viral vectors. Specifically, vectors for the transfection of genetic material have been developed from whole viruses, including adenoviruses and retroviruses. However, only a limited amount of biologically active materials can be placed inside of a

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viral capsule. Moreover, in the case of biologically active materials which comprise genetic material, undesired interaction of the viral carrier may occur with the encapsulated genetic material and the patient.

To minimize the potential interactions associated with viruses, attempts have been made to use only certain components of a virus. This is difficult to achieve in vivo inasmuch as the virus components must be able to recognize and reach the targeted cells. Despite extensive work, a successfully targeted, viral-mediated vector for the delivery of biologically active materials into cells in vivo has not been adequately achieved.

As noted above, liposomes have been used as a carrier for the intracellular delivery of biologically active agents, including genetic material. One of the original methods for the use of liposomes as carriers for biologically active agents is disclosed in Szoka and Papahadjopoulos, *Ann. Rev. Biophysic. Bioeng.*, Vol. 9, pp. 467–508 (1980). The disclosed method involves the preparation of liposomes by the addition of an aqueous solution of genetic material to phospholipids which are dissolved in ether. Evaporation of the ether phase provides genetic material encapsulated in lipid vesicles.

Another method for encapsulating biologically active agents in liposomes involves the extrusion of dehydration-rehydration vesicles. Other methods, in addition to those described above, are known for the encapsulation by liposomes of biologically active agents.

More recently, liposomes have been developed from cationic lipids, such as N-[1-(2,3-dioleoyloxy)propyl]-N,N, N-trimethylammonium chloride ("DOTMA") or lipids which comprise cationic polymers, for example, polysine. See, e.g., Xiaohuai and Huang, *Biochimica et Biophysica Acta*, Vol. 1189, pp. 195–203 (1994). Liposomes which are prepared from cationic materials (referred to hereinafter as "cationic liposomes") have been developed, inter alia, to transfect cells with genetic material, including DNA. It is believed that the cationic liposomes bind with the negatively charged phosphate group(s) of the nucleotides in DNA. Studies have shown that cationic liposomes mediate transfection of cells with genetic material in vitro more efficiently than other carriers, for example, cationic polymers. In addition, in vitro studies have shown also that cationic liposomes provide improved transfection of cells relative to other delivery methods, including electroporation and calcium phosphate precipitation.

However, the currently available cationic lipids and cationic liposomes are generally ineffective for the intracellular delivery of biologically active agents in vivo. Moreover, they are generally ineffective for the intracellular delivery of biologically active agents in serum. This is a serious drawback inasmuch as cells require serum for viability. In fact, it is generally necessary to remove serum from tissue culture baths during gene transfection studies involving cationic lipids and cationic liposomes. After transfection, the serum is replaced. This involves additional processing steps which render transfection of cells with cationic lipids and cationic liposomes complex and cumbersome.

New and/or better cationic lipids useful, inter alia, for the intracellular delivery of bioactive agents are needed. The present invention is directed to this as well as other important ends.

SUMMARY OF THE INVENTION

The present invention is directed to cationic lipids which comprise at least one, and preferably at least two, cationic

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groups and which may be useful for the intracellular delivery of bioactive agents.

Specifically, in one embodiment, the present invention relates to a cationic lipid compound of the formula

$$Y_1 - (R_1 - X_1)_x - R_2 - [Y_2 - R_3]_y - (X_1 - R_1)_x - Y_1 \qquad (I)$$

with substituent $(R_4 - Y_3)_z$ on R_2

wherein:

each of x, y and z is independently an integer from 0 to about 100;

each X_1 is independently —O—, —S—, —NR$_5$—, —C(=X_2)—, —C(=X_2)—N(R$_5$)—, —N(R$_5$)—C(=X_2)—, —C(=X_2)—O—, —O—C(=X_2)— or —X$_2$—(R$_5$X$_2$)P(=X$_2$)—X$_2$—;

each X_2 is independently O or S;

each Y_1 is independently a phosphate residue, N(R$_6$)$_a$—, S(R$_6$)$_a$—, P(R$_6$)$_a$— or —CO$_2$R$_6$, wherein a is an integer from 1 to 3;

each Y_2 is independently —N(R$_6$)$_b$—, —S(R$_6$)$_b$— or —P(R$_6$)$_b$—, wherein b is an integer from 0 to 2;

each Y_3 is independently a phosphate residue, N(R$_6$)$_a$—, S(R$_6$)$_a$—, P(R$_6$)$_a$— or —CO$_2$R$_6$, wherein a is an integer from 1 to 3;

each of R$_1$, R$_2$, R$_3$ and R$_4$ is independently alkylene of 1 to about 20 carbons;

each R$_5$ is independently hydrogen or alkyl of 1 to about 10 carbons; and

each R$_6$ is independently —[R$_7$—X$_3$]$_c$—R$_8$ or —R$_9$—[X$_4$—R$_{10}$]$_d$—Q, wherein:

each of c and d is independently an integer from 0 to about 100;

each Q is independently a phosphate residue, —N(R$_{11}$)$_q$, —S(R$_{11}$)$_q$, —P(R$_{11}$)$_q$ or —CO$_2$R$_6$, wherein q is an integer from 1 to 3;

each of X$_3$ and X$_4$ is independently —O—, —S—, —NR$_5$—, —C(=X$_2$)—, —C(=X$_2$)—N(R$_5$)—, —N(R$_5$)—C(=X$_2$)—, —C(=X$_2$)—O—, —O—C(=X$_2$)— or —X$_2$—(R$_5$X$_2$)P(=X$_2$)—X$_2$—;

each R$_7$ is independently alkylene of 1 to about 20 carbons;

each R$_8$ is independently hydrogen or alkyl of 1 to about 60 carbons;

each of R$_9$ and R$_{10}$ is independently alkylene of 1 to about 20 carbons; and

each R$_{11}$ is independently —[R$_7$—X$_3$]$_c$—R$_8$ or —R$_9$—[X$_4$—R$_{10}$]$_d$—W, wherein:

each W is independently a phosphate residue, —N(R$_{12}$)$_w$, —S(R$_{12}$)$_w$, —P(R$_{12}$)$_w$ or —CO$_2$R$_6$, wherein w is an integer from 1 to 3; and

R$_{12}$ is —[R$_7$—X$_3$]$_c$—R$_8$; with the proviso that the compound of formula (I) comprises at least one, and preferably at least two, quaternary salts.

In another embodiment, the invention relates to a cationic lipid compound of the formula

$$Y_1 - R_1 - Y_1 \qquad (II)$$

wherein:

each Y$_1$ is independently a phosphate residue, N(R$_2$)$_a$—, S(R$_2$)$_a$—, P(R$_2$)$_a$— or —CO$_2$R$_2$, wherein a is an integer from 1 to 3;

R$_1$ is alkylene of 1 to about 60 carbons containing 0 to about 30 —O—, —S—, —NR$_3$— or —X$_2$—(R$_3$X$_2$)P(=X$_2$)—X$_2$— heteroatoms or heteroatom groups;

R$_2$ is a residue of the formula —R$_4$—[(X$_1$—R$_5$)$_x$—Y$_2$]$_y$—R$_6$, wherein:

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each of x and y is independently an integer from 0 to about 100;

each X$_1$ is independently a direct bond, —O—, —S—, —NR$_3$—, —C(=X$_2$)—, —C(=X$_2$)—N(R$_3$)—, —N(R$_3$)—C(=X$_2$)—, —C(=X$_2$)—O—, —O—C(=X$_2$)— or —X$_2$—(R$_3$X$_2$)P(=X$_2$)—X$_2$—;

each X$_2$ is independently O or S;

each Y$_2$ is independently —S(R$_2$)$_b$—, —N(R$_2$)$_b$— or —P(R$_2$)$_b$—, wherein b is an integer from 0 to 2;

each R$_3$ is independently hydrogen or alkyl of 1 to about 10 carbons;

each of R$_4$ and R$_5$ is independently a direct bond or alkylene of 1 to about 30 carbons containing 0 to about 15 —O—, —S—, —NR$_3$— or —X$_2$—(R$_3$X$_2$)P(=X$_2$)—X$_2$— heteroatoms or heteroatom groups; and

each R$_6$ is independently hydrogen or alkyl of 1 to about 60 carbons containing 0 to about 30 —O—, —S—, —NR$_3$— or —X$_2$—(R$_3$X$_2$)P(=X$_2$)—X$_2$— heteroatoms or heteroatom groups; with the proviso that the compound of formula (II) comprises at least one, and preferably at least two, quaternary salts.

In yet another embodiment, the present invention relates to a cationic lipid compound of the formula

$$Y_1 - (R_1 - X_1)_x - R_2 - [Y_2 - R_3]_y - (X_1 - R_1)_x - Y_1 \qquad (III)$$

with branches $(R_4 - Y_3)_z$, $(R_1 - X_1)_x$, R$_2$, $Y_1 - (R_1 - X_1)_x - [R_3 - Y_2]_y - R_2 - (X_1 - R_1)_x - Y_1$, and $(R_4 - Y_3)_z$

wherein:

each of x, y and z is independently an integer from 0 to about 100;

each X$_1$ is independently —O—, —S—, —NR$_5$—, —C(=X$_2$)—, —C(=X$_2$)—N(R$_5$)—, —N(R$_5$)—C(=X$_2$)—, —C(=X$_2$)—O—, —O—C(=X$_2$)— or —X$_2$—(R$_5$X$_2$)P(=X$_2$)—X$_2$—;

each X$_2$ is independently O or S;

each Y$_1$ is independently —O—, —N(R$_6$)$_a$—, —S(R$_6$)$_a$— or —P(R$_6$)$_a$—, wherein a is an integer from 0 to 2;

each Y$_2$ is independently —N(R$_6$)$_a$—, —S(R$_6$)$_a$— or —P(R$_6$)$_a$—, wherein a is an integer from 0 to 2;

each Y$_3$ is independently a phosphate residue, N(R$_6$)$_b$—, S(R$_6$)$_b$—, P(R$_6$)$_b$— or —CO$_2$R$_6$, wherein b is an integer from 1 to 3;

each of R$_1$, R$_2$, R$_3$ and R$_4$ is independently alkylene of 1 to about 20 carbons;

each R$_5$ is independently hydrogen or alkyl of 1 to about 10 carbons; and

each R$_6$ is independently —[R$_7$—X$_3$]$_c$—R$_8$ or —R$_9$—[X$_4$—R$_{10}$]$_d$—Q, wherein:

each of c and d is independently an integer from 0 to about 100;

each Q is independently a phosphate residue, —N(R$_{11}$)$_q$, —S(R$_{11}$)$_q$, —P(R$_{11}$)$_q$ or —CO$_2$R$_{11}$, wherein q is an integer from 1 to 3;

each of X$_3$ and X$_4$ is independently —O—, —S—, —NR$_5$—, —C(=X$_2$)—, —C(=X$_2$)—N(R$_5$)—, —N(R$_5$)—C(=X$_2$)—, —C(=X$_2$)—O—, —O—C(=X$_2$)— or —X$_2$—(R$_5$X$_2$)P(=X$_2$)—X$_2$—;

each R$_7$ is independently alkylene of 1 to about 20 carbons;

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each R_8 is independently hydrogen or alkyl of 1 to about 60 carbons;

each of R_9 and R_{10} is independently alkylene of 1 to about 20 carbons; and

each R_{11}, is independently —$[R_7$—$X_3]_c$—R_8 or —R_9—$[X_4$—$R_{10}]_d$—W, wherein:

each W is independently a phosphate residue, —$N(R_{12})_w$, —$S(R_{12})_w$, —$P(R_{12})_w$ or —CO_2R_{12}, wherein w is an integer from 1 to 3; and

R_{12} is —$[R_7$—$X_3]_c$—R_8; with the proviso that the compound of formula (III) comprises at least one, and preferably at least two, quaternary salts.

Cationic lipid compounds which comprise at least one, and preferably at least two, cationic groups are also the subject of the present invention.

Another aspect of the present invention are cationic lipid compositions which are composed of cationic lipid compounds that comprise at least one, and preferably at least two, cationic groups.

Yet another aspect of the present invention is a cationic lipid formulation for the intracellular delivery of a bioactive agent. The formulation comprises, in combination with a bioactive agent, a cationic lipid compound that comprises at least one, and preferably at least two cationic groups.

Still another aspect of the present invention relates to a process for the preparation of a cationic lipid formulation for the intracellular delivery of a bioactive agent. The process comprises combining together a bioactive agent and a cationic lipid composition which comprises a cationic lipid compound having at least one, and preferably at least two, cationic groups.

Also encompassed by the present invention is a method for delivering intracellularly a bioactive agent. The method comprises contacting cells with a cationic lipid compound having at least one, and preferably at least two, cationic groups and a bioactive agent.

These and other aspects of the invention will become more apparent from the present specification and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1** and **2** are graphical representations of the amount of protein expressed in transfection experiments involving cationic lipid compounds of the present invention and compounds disclosed in the prior art.

DETAILED DESCRIPTION OF THE INVENTION

As employed above and throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings.

"Alkyl" refers to an aliphatic hydrocarbon group which may be straight or branched having 1 to about 60 carbon atoms in the chain. "Lower alkyl" refers to an alkyl group having 1 to about 8 carbon atoms. "Higher alkyl" refers to an alkyl group having about 10 to about 20 carbon atoms. The alkyl group may be optionally substituted with one or more alkyl group substituents which may be the same or different, where "alkyl group substituent" includes halo, aryl, hydroxy, alkoxy, aryloxy, alkyloxy, alkylthio, arylthio, aralkyloxy, aralkylthio, carboxy alkoxycarbonyl, oxo and cycloalkyl. There may be optionally inserted along the alkyl group one or more oxygen, sulphur or substituted or unsubstituted nitrogen atoms, wherein the nitrogen substituent is lower alkyl. "Branched" refers to an alkyl group in which a lower alkyl group, such as methyl, ethyl or propyl, is attached to a linear alkyl chain. Exemplary alkyl groups

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include methyl, ethyl, i-propyl, n-butyl, t-butyl, n-pentyl, heptyl, octyl, decyl, dodecyl, tridecyl, tetradecyl, pentadecyl and hexadecyl. Preferred alkyl groups include the lower alkyl groups of 1 to about 4 carbons and the higher alkyl groups of about 12 to about 16 carbons. Preferred alkyl groups include also alkyl groups which are substituted with one or more halo atoms. Fluoroalkyl groups are preferred among the halo-substituted alkyl groups, including, for example, fluoroalkyl groups of the formula $CF_3(CF_2)_n$ $(CH_2)_m$—, wherein each of m and n is independently an integer from 0 to about 22. Exemplary fluoroalkyl groups include perfluoromethyl, perfluoroethyl, perfluoropropyl, perfluorobutyl, perfluorocyclobutyl, perfluoropentyl, perfluorohexyl, perfluoroheptyl, perfluorooctyl, perfluorononyl, perfluorodecyl, perfluoroundecyl and perfluorododecyl.

"Alkenyl" refers to an alkyl group containing at least one carbon-carbon double bond. The alkenyl group may be optionally substituted with one or more "alkyl group substituents". Exemplary alkenyl groups include vinyl, allyl, n-pentenyl, decenyl, dodecenyl, tetradecadienyl, heptadec-8-en-1-yl and heptadec-8,11-dien-1-yl.

"Alkynyl" refers to an alkyl group containing a carbon-carbon triple bond. The alkynyl group may be optionally substituted with one or more "alkyl group substituents". Exemplary alkynyl groups include ethynyl, propargyl, n-pentynyl, decynyl and dodecynyl. Preferred alkynyl groups include the lower alkynyl groups.

"Cycloalkyl" refers to a non-aromatic mono- or multicyclic ring system of about 4 to about 10 carbon atoms. The cycloalkyl group may be optionally partially unsaturated. The cycloalkyl group may be also optionally substituted with an aryl group substituent, oxo and/or alkylene. Preferred monocyclic cycloalkyl rings include cyclopentyl, cyclohexyl and cycloheptyl. Preferred multicyclic cycloalkyl rings include adamantyl, octahydronaphthyl, decalin, camphor, camphane, noradamantyl, bicyclo[2.2.2.] oct-5-ene, cis-5-norbornene, 5-norbornene, (1R)-(−)-myrtentane, norbornane and anti-3-oxo-tricyclo[$2.2.1.0^{2,6}$] heptane.

"Aryl" refers to an aromatic carbocyclic radical containing about 6 to about 10 carbon atoms. The aryl group may be optionally substituted with one or more aryl group substituents which may be the same or different, where "aryl group substituent" includes alkyl, alkenyl, alkynyl, aryl, aralkyl, hydroxy, alkoxy, aryloxy, aralkoxy, carboxy, aroyl, halo, nitro, trihalomethyl, cyano, alkoxycarbonyl, aryloxycarbonyl, aralkoxycarbonyl, acyloxy, acylamino, aroylamino, carbamoyl, alkylcarbamoyl, dialkylcarbamoyl, rylthio, alkylthio, alkylene and —NRR', where R and R' are each independently hydrogen, alkyl, aryl and aralkyl. Exemplary aryl groups include substituted or unsubstituted phenyl and substituted or unsubstituted naphthyl.

"Acyl" refers to an alkyl-CO— group wherein alkyl is as previously described. Preferred acyl groups comprise alkyl of 1 to about 30 carbon atoms. Exemplary acyl groups include acetyl, propanoyl, 2-methylpropanoyl, butanoyl and palmitoyl.

"Aroyl" means an aryl-CO— group wherein aryl is as previously described. Exemplary aroyl groups include benzoyl and 1- and 2-naphthoyl.

"Alkoxy" refers to an alkyl-O— group wherein alkyl is as previously described. Exemplary alkoxy groups include methoxy, ethoxy, n-propoxy, i-propoxy, n-butoxy and heptoxy.

"Aryloxy" refers to an aryl-O— group wherein the aryl group is as previously described. Exemplary aryloxy groups include phenoxy and naphthoxy.

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"Alkylthio" refers to an alkyl-S— group wherein alkyl is as previously described. Exemplary alkylthio groups include methylthio, ethylthio, i-propylthio and heptylthio.

"Arylthio" refers to an aryl-S— group wherein the aryl group is as previously described. Exemplary arylthio groups include phenylthio and naphthylthio.

"Aralkyl" refers to an aryl-alkyl- group wherein aryl and alkyl are as previously described. Exemplary aralkyl groups include benzyl, phenylethyl and naphthylmethyl.

"Aralkyloxy" refers to an aralkyl-O— group wherein the aralkyl group is as previously described. An exemplary aralkyloxy group is benzyloxy.

"Aralkylthio" refers to an aralkyl-S— group wherein the aralkyl group is as previously described. An exemplary aralkylthio group is benzylthio.

"Dialkylamino" refers to an —NRR' group wherein each of R and R' is independently an alkyl group as previously described. Exemplary alkylamino groups include ethylmethylamino, dimethylamino and diethylamino.

"Alkoxycarbonyl" refers to an alkyl-O—CO— group. Exemplary alkoxycarbonyl groups include methoxycarbonyl, ethoxycarbonyl, butyloxycarbonyl and t-butyloxycarbonyl.

"Aryloxycarbonyl" refers to an aryl-O—CO— group. Exemplary aryloxycarbonyl groups include phenoxy- and naphthoxy-carbonyl.

"Aralkoxycarbonyl" refers to an aralkyl-O—CO— group. An exemplary aralkoxycarbonyl group is benzyloxycarbonyl.

"Carbamoyl" refers to an H_2N—CO— group.

"Alkylcarbamoyl" refers to a R'RN—CO— group wherein one of R and R' is hydrogen and the other of R and R' is alkyl as previously described.

"Dialkylcarbamoyl" refers to R'RN—CO— group wherein each of R and R' is independently alkyl as previously described.

"Acyloxy" refers to an acyl-O— group wherein acyl is as previously described.

"Acylamino" refers to an acyl-NH— group wherein acyl is as previously described.

"Aroylamino" refers to an aroyl-NH— group wherein aroyl is as previously described.

"Alkylene" refers to a straight or branched bivalent aliphatic hydrocarbon group having from 1 to about 30 carbon atoms. The alkylene group may be straight, branched or cyclic. The alkylene group may be also optionally unsaturated and/or substituted with one or more "alkyl group substituents." There may be optionally inserted along the alkylene group one or more oxygen, sulphur or substituted or unsubstituted nitrogen atoms, wherein the nitrogen substituent is alkyl as previously described. Exemplary alkylene groups include methylene (—CH_2—), ethylene (—CH_2CH_2—), propylene (—$(CH_2)_3$—), cyclohexylene (—C_6H_{10}—), —CH=CH—CH=CH—, —CH=CH—CH_2—, —$(CF_2)_n(CH_2)_m$—, wherein n is an integer from about 1 to about 22 and m is an integer from 0 to about 22, —$(CH_2)_n$—N(R)—$(CH_2)_m$—, wherein each of m and n is independently an integer from 0 to about 30 and R is hydrogen or alkyl, methylenedioxy (—O—CH_2—O—) and ethylenedioxy (—O—$(CH_2)_2$—O—). It is preferred that the alkylene group has about 2 to about 3 carbon atoms.

"Halo" or "halide" refers to fluoride, chloride, bromide or iodide.

"Heteroatom group" refers to a radical which contains at least one heteroatom.

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"Amino Acid" refers to a naturally occurring or synthetic amino acid.

"Polypeptide" refers to a biologically active series of two or more amino acid residues bonded by peptide linkages. Polypeptides having about 3 to about 40 amino acid residues are preferred.

"Phosphate residue" refers to a substituent group which is derived from phosphoric acid (O=P(OH)$_3$). Preferably, the phosphate residue is an ester of phosphoric acid which is substituted with one or more alkyl and/or alkenyl groups. Preferred phosphate esters include phospholipids. Preferred among the phospholipids are phosphoglycerides, with diacylglycerol phosphates being especially preferred. An exemplary diacylglycerol phosphate is 1,2-dioleoylglycero-3-phosphoethyl.

"Quaternary salt" refers to a type of ammonium, sulfonium or phosphonium compound in which the hydrogen atoms of the ammonium, sulfonium or phosphonium ion are replaced by alkyl groups. With respect to quaternary ammonium and phosphonium compounds, the molecular structure includes a nitrogen or phosphorous atom joined to four organic groups, for example, alkyl groups. The molecular structure of a quaternary sulfonium compound includes a sulfur atom joined to three organic groups. These molecular structures are positively charged and are generally referred to as cations or cationic groups. The cations are typically, although not necessarily, associated with a negatively charged acid radical. The negatively charged radical is generally referred to as an anion or an anionic group. Exemplary anions include, for example, halides. Quaternary salts are generally the product of the final stage of alkylation of nitrogen, sulfur or phosphorous.

"Lipid" refers to a synthetic or naturally-occurring amphipathic compound which comprises a hydrophilic component and a hydrophobic component. Lipids include, for example, fatty acids, neutral fats, phosphatides, glycolipids, aliphatic alcohols and waxes, terpenes and steroids.

"Cationic lipid compound" refers to a lipid which comprises a cationic group and which functions generally as a positively charged ion, for example, in solution. Preferred cationic lipid compounds are lipids which comprise at least one cationic group, with lipids which comprise at least two or more cationic groups being more preferred.

"Cationic group" refers to a group which is positively charged. Preferred cationic groups include the positively charged ions of quaternary salts. Exemplary quaternary salts are ammonium, phosphonium and sulfonium salts.

"Counter ion" refers to an anion. An anion which is "pharmaceutically-acceptable" is substantially non-toxic and does not render the associated cation pharmaceutically unacceptable.

"Cationic lipid composition" refers to a composition which comprises a cationic lipid compound. Exemplary cationic lipid compositions include suspensions, emulsions, vesicular compositions and hexagonal H II phase structures. "Cationic lipid formulation" refers to a composition which comprises a cationic lipid compound and a bioactive agent.

"Charge density" refers to charge per unit mass or volume.

"Vesicle" or "vesicular species" refers to a spherical entity which is characterized by the presence of an internal void. Preferred vesicles or vesicular species are formulated from lipids, including the cationic lipid compounds of the present invention. In any given vesicle or vesicular species, the lipids may be in the form of a monolayer or bilayer, and the

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mono- or bilayer lipids may be used to form one or more mono- or bilayers. In the case of more than one mono- or bilayer, the mono- or bilayers are generally,concentric. The lipid vesicles or vesicular species include such entities commonly referred to as liposomes, micelles and the like. Thus, the lipids may be used to form a unilamellar vesicle (comprised of one monolayer or bilayer), an oligolamellar vesicle (comprised of about two or about three monolayers or bilayers) or a multilamellar vesicle (comprised of more than about three monolayers or bilayers). The internal void of the vesicles are generally filled with a liquid, including, for example, an aqueous liquid, a gas, a gaseous precursor, and/or a solid material, including, for example, a bioactive agent.

"Cationic vesicle" or "cationic vesicular composition" refers to a vesicle or vesicular species which is formulated from a cationic lipid compound.

"Cationic vesicle formulation" refers to a composition of a vesicle or vesicular species and a bioactive agent.

"Liposome" refers to a generally spherical cluster or aggregate of amphipathic compounds, including lipid compounds, typically in the form of one or more concentric layers, for example, bilayers.

"Emulsion" refers to a lipoidal mixture of two or more liquids and is generally in the form of a colloid. The lipids may be heterogeneously dispersed throughout the emulsion. Alternatively, the lipids may be aggregated in the form of, for example, clusters or layers, including mono- or bilayers.

"Suspension" refers to a mixture of finely divided colloidal particles floating in a liquid.

"Hexagonal H II phase structure" refers to a generally tubular aggregation of lipids in liquid media, for example, aqueous media, in which the hydrophilic portion(s) of the lipids generally face inwardly in association with a liquid environment inside the tube. The hydrophobic portion(s) of the lipids generally radiate outwardly and the complex assumes the shape of a hexagonal tube. A plurality of tubes is generally packed together in the hexagonal phase structure.

"Patient", as used herein, refers to animals, including mammals, preferably humans.

"Bioactive agent" refers to a substance which is capable of exerting a biological effect in vitro and/or in vivo. The biological effect is preferably therapeutic in nature. As used herein, "bioactive agent" refers also to substances which are used in connection with an application which is diagnostic in nature, such as in methods for diagnosing the presence or absence of a disease in a patient. The bioactive agents may be neutral or positively or negatively charged. Preferably, the bioactive agents are negatively charged. Examples of suitable bioactive agents include pharmaceuticals and drugs, synthetic organic molecules, proteins, vitamins, steroids, polyanions, nucleosides, nucleotides, polynucleotides and diagnostic agents, such as contrast agents for use in connection with magnetic resonance imaging, ultrasound or computed tomography of a patient.

"Anionic group" refers to a group which is negatively charged. Preferred anionic groups include phosphate (PO_4^-) groups.

"Anionic bioactive agent" refers to a bioactive agent that comprises at least one anionic group. Certain genetic materials, for example, polynucleotides, are exemplary anionic bioactive agents.

"Genetic material" refers generally to nucleotides and polynucleotides, including deoxyribonucleic acid (DNA)

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and ribonucleic acid (RNA). The genetic material may be made by synthetic chemical methodology known to one of ordinary skill in the art, or by the use of recombinant technology, or by a combination of the two. The DNA and RNA may optionally comprise unnatural nucleotides and may be single or double stranded. "Genetic material" refers also to sense and anti-sense DNA and RNA, that is, a nucleotide sequence which is complementary to a specific sequence of nucleotides in DNA and/or RNA.

"Pharmaceutical" or "drug" refers to any therapeutic or prophylactic agent which is used in the prevention, diagnosis, alleviation, treatment or cure of a malady, affliction, disease or injury in a patient. Therapeutically useful polynucleotides and polypeptides are included within the definition of drug.

"In combination with" refers to the incorporation of a bioactive agent with a cationic lipid compound of the present invention. The cationic lipid compound can be combined with the bioactive agent in any of a variety of different ways. For example, when the cationic lipid compound is in the form of a cationic vesicle or a cationic vesicular composition, the bioactive agent may be entrapped within the internal void of the vesicle. It is also contemplated that the bioactive agent may be integrated within the layer(s) or wall(s) of the vesicle, for example, by being interspersed among lipids which are contained within the vesicular layer(s) or wall(s). In addition, it is contemplated that the bioactive agent may be located on the surface of a vesicle. In this case, the bioactive agent may interact chemically with the surface of the vesicle and remain substantially adhered thereto. Such interaction may take the form of, for example, electrostatic interactions, hydrogen bonding, van der Waal's forces or covalent bonding. Alternatively, or in addition to, the bioactive agent may interact with the surface of the vesicle in a limited manner. Such limited interaction would permit migration of the bioactive agent, for example, from the surface of a first vesicle to the surface of a second vesicle.

"Intracellular" or "intracellularly" refers to the area within the plasma membrane of a cell, including the protoplasm, cytoplasm and/or nucleoplasm. "Intracellular delivery" refers to the delivery of a bioactive agent into the area within the plasma membrane of a cell.

"Cell" refers to any one of the minute protoplasmic masses which make up organized tissue, comprising a mass of protoplasm surrounded by a membrane, including nucleated and unnucleated cells and organelles.

"Immune competence" refers to the ability of the immune system to protect against pathogens or infectious agents.

The present invention is directed, in part, to a new class of cationic lipid compounds which are highly useful in connection with the intracellular delivery of one or more bioactive agents. The new class of lipids are described in more detail below.

Specifically, in one embodiment, the present invention relates to a cationic lipid compound of the formula

$$Y_1 - (R_1 - X_1)_x - R_2 - [Y_2 - R_3]_y - (X_1 - R_1)_x - Y_1 \overset{\overset{\displaystyle (R_4 - Y_3)_z}{|}}{} \quad (I)$$
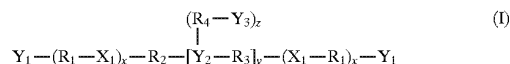
wherein:

each of x, y and z is independently an integer from 0 to about 100;

each X_1 is independently $-O-$, $-S-$, $-NR_5-$, $-C(=X_2)-$, $-C(=X_2)-N(R_5)-$, $-N(R_5)-C(=X_2)-$, $-C(=X_2)-O-$, $-O-C(=X_2)-$ or $-X_2-(R_5X_2)P(=X_2)-X_2-$;

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each X_2 is independently O or S;

each Y_1 is independently a phosphate residue, $N(R_6)_a$—, $S(R_6)_a$—, $P(R_6)_a$— or —CO_2R_6, wherein a is an integer from 1 to 3;

each Y_2 is independently —$N(R_6)_b$—, —$S(R_6)_b$— or —$P(R_6)_b$—, wherein b is an integer from 0 to 2;

each Y_3 is independently a phosphate residue, $N(R_6)_a$—, $S(R_6)_a$—, $P(R_6)_a$— or —CO_2R_6, wherein a is an integer from 1 to 3;

each of R_1, R_2, R_3 and R_4 is independently alkylene of 1 to about 20 carbons;

each R_5 is independently hydrogen or alkyl of 1 to about 10 carbons; and

each R_6 is independently —$[R_7—X_3]_c—R_8$ or —R_9—$[X_4—R_{10}]_d$—Q, wherein:

    each of c and d is independently an integer from 0 to about 100;

    each Q is independently a phosphate residue, —$N(R_{11})_q$, —$S(R_{11})_q$, —$P(R_{11})_q$ or —CO_2R_{11}, wherein q is an integer from 1 to 3;

    each of X_3 and X_4 is independently —O—, —S—, —NR_5—, —$C(=X_2)$—, —$C(=X_2)$—$N(R_5)$—, —$N(R_5)$—$C(=X_2)$—, $C(=X_2)$—O—, —O—C$(=X_2)$— or —X_2—$(R_5X_2)P(=X_2)$—X_2—;

    each R_7 is independently alkylene of 1 to about 20 carbons;

    each R_8 is independently hydrogen or alkyl of 1 to about 60 carbons;

    each of R_9 and R_{10} is independently alkylene of 1 to about 20 carbons; and

    each R_{11} is independently —$[R_7—X_3]_c—R_8$ or —R_9—$[X_4—R_{10}]_d$—W, wherein:

        each W is independently a phosphate residue, —$N(R_{12})_w$, —$S(R_{12})_w$, —$P(R_{12})_w$ or —CO_2R_{12}, wherein w is an integer from 1 to 3; and

        R_{12} is —$[R_7—X_3]_c—R_8$; with the proviso that the compound of formula (I) comprises at least one, and preferably at least two, quaternary salts.

In the above formula (I), each of x, y and z is independently an integer from 0 to about 100. Preferably, each of x, y and z is independently an integer of from 0 to about 50, with integers from 0 to about 20 being more preferred. Even more preferably, each of x, y and z is independently an integer from 0 to about 10, with integers from 0 to about 5 being still more preferred. In certain particularly preferred embodiments, x is 1, y is 2 or 3 and z is 0 or 1.

In the above formula (I), each X_1 is independently —O—, —S—, —NR_5—, —$C(=X_2)$—, —$C(=X_2)$—$N(R_5)$—, —$N(R_5)$—$C(=X_2)$—, —$C(=X_2)$—O—, —O—C$(=X_2)$— or —X_2—$(R_5X_2)P(=X_2)$—X_2—. Preferably, each X_1 is independently —$C(=O)$—NR_5—, —NR_5—C$(=O)$—, —$C(=O)$—O— or —O—$C(=O)$—.

Each X_2 in the definitions of X_1, X_3 and X_4 above is independently O or S. Preferably, X_2 is O.

In the above formula (I), each Y_1 is independently a phosphate residue, $N(R_6)_a$—, $S(R_6)_a$—, $P(R_6)_a$— or —CO_2R_6, wherein a is an integer from 1 to 3. Preferably, each Y_1 is independently a phosphate residue, $N(R_6)_a$— or —CO_2R_6, wherein a is 2 or 3. Preferably, a is 3.

Each Y_2 in formula (I) above is independently —$N(R_6)_b$—, —$S(R_6)_b$— or —$P(R_6)_b$—, wherein b is an integer from 0 to 2. Preferably, Y_2 is —$N(R_6)_b$—, wherein b is 1 or 2.

In the above formula (I), each Y_3 is independently a phosphate residue, $N(R_6)_a$—, $S(R_6)_a$—, $P(R_6)_a$— or —CO_2R_6, wherein a is an integer from 1 to 3. Preferably,

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each Y_3 is independently a phosphate residue, $N(R_6)_a$— or —CO_2R_6, wherein a is 2 or 3. Preferably, a is 3.

In the above formula (I), each of R_1, R_2, R_3 and R_4 is independently alkylene of 1 to about 20 carbons. Preferably, each of R_1, R_2, R_3 and R_4 is independently straight chain alkylene of 1 to about 10 carbons or cycloalkylene of about 4 to about 10 carbons. More preferably, each of R_1, R_2, R_3 and R_4 is independently straight chain alkylene of 1 to about 4 carbons or cycloalkylene of about 5 to about 7 carbons. Even more preferably, each of R_1, R_2, R_3 and R_4 is independently methylene, ethylene or cyclohexylene.

In the above definitions of X_1, X_3 and X_4, each R_5 is independently hydrogen or alkyl of 1 to about 10 carbons. Preferably, each R_5 is independently hydrogen or alkyl of 1 to about 4 carbons. More preferably, R_5 is hydrogen.

In the above definitions of Y_1, Y_2 and Y_3, each R_6 is independently —$[R_7—X_3]_c—R_8$ or —R_9—$[X_4—R_{10}]_d$—Q, wherein each of c and d is independently an integer from 0 to about 100. Preferably, each of c and d is independently an integer from 0 to about 50, with integers from 0 to about 20 being more preferred. Even more preferably, each of c and d is independently an integer from 0 to about 10, with integers from 0 to about 5 being still more preferred. In certain particularly preferred embodiments, c is 0 or 1 and d is 1.

Each Q in R_6 above is independently a phosphate residue, —$N(R_{11})_q$, —$S(R_{11})_q$ or —$P(R_{11})_q$ or —CO_2R_{11}, wherein q is an integer from 1 to 3. Preferably, each Q is independently a phosphate residue, —$N(R_{11})_q$ or —CO_2R_{11}, wherein q is 2 or 3. Preferably, q is 3.

Also in the above definition of R_6, each of X_3 and X_4 is independently —O—, —S—, —NR_5—, —$C(=X_2)$—, —$C(=X_2)$—$N(R_5)$—, —$N(R_5)$—$C(=X_2)$—, —$C(=X_2)$—O—, —O—$C(=X_2)$— or —X_2—$(R_5X_2)P(=X_2)$—X_2—, wherein each of X_2 and R_5 is independently as previously described. Preferably, each of X_3 and X_4 is independently —$C(=O)$—NR_6—, —NR_5—C$(=O)$—, —$C(=O)$—O— or —O—$C(=O)$—.

In the definitions of R_6, R_{11} and R_{12} above, each R_7 is independently alkylene of 1 to about 20 carbons. Preferably, each R_7 is independently alkylene of 1 to about 10 carbons, with alkylene of 1 to about 4 carbons being preferred. More preferably, each R_7 is independently methylene or ethylene.

Also in the definitions of R_6, R_{11} and R_{12} above, each R_8 is independently hydrogen or alkyl of 1 to about 60 carbons. Preferably, each R_8 is independently hydrogen or alkyl of 1 to about 40 carbons, with hydrogen or alkyl of 1 to about 20 carbons being more preferred. Even more preferred, each R_8 is independently hydrogen or alkyl of 1 to about 16 carbons. In certain particularly preferred embodiments, each R_8 is independently hydrogen, methyl, dodecyl or hexadecyl.

Each of R_9 and R_{10} in the definitions of R_6 and R_{11} above is independently alkylene of 1 to about 20 carbons. Preferably, each of R_9 and R_{10} is independently alkylene of 1 to about 10 carbons. More preferably, each of R_9 and R_{10} is independently alkylene of 1 to about 4 carbons. Even more preferably, each of R_9 and R_{10} is independently methylene or ethylene.

Each R_{11} in Q above is independently —$[R_7—X_3]_c—R_8$ or —R_9—$[X_4—R_{10}]_d$—W, wherein each of c, d, X_3, X_4, R_7, R_8, R_9 and R_{10} is independently as previously described.

Each W in R_{11} above is independently a phosphate residue, —$N(R_{12})_w$, —$S(R_{12})_w$, —$P(R_{12})_w$ or —CO_2R_{12}, wherein w is an integer from 1 to 3. Preferably, W is a phosphate residue, —$N(R_{12})_w$ or —CO_2R_{12}, wherein w is 2 or 3. Preferably, w is 3.

In the above definition of W, R_{12} is —$[R_7—X_3]_c—R_8$, wherein each of c, X_3, R_7 and R_8 is independently as previously described.

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In another embodiment of the present invention, there is provided a cationic lipid compound of the formula

$$Y_1—R_1—Y_1 \qquad (II)$$

wherein:

each Y_1 is independently a phosphate residue, $N(R_2)_a—$, $S(R_2)_a—$, $P(R_2)_a—$ or $—CO_2R_2$, wherein a is an integer from 1 to 3;

R_1 is alkylene of 1 to about 60 carbons containing 0 to about 30 $—O—$, $—S—$, $—NR_3—$ or $—X_2—(R_3X_2)P(=X_2)—X_2—$ heteroatoms or heteroatom groups;

R_2 is a residue of the formula $—R_4—[(X_1—R_5)_x—Y_2]_y—R_6$, wherein:

each of x and y is independently an integer from 0 to about 100;

each X_1 is independently a direct bond, $—O—$, $—S—$, $—NR_3—$, $—C(=X_2)—$, $—C(=X_2)—N(R_3)—$, $—N(R_3)—C(=X_2)—$, $—C(=X_2)—O—$, $—O—C(=X_2)—$ or $—X_2—(R_3X_2)P(=X_2)—X_2—$;

each X_2 is independently O or S;

each Y_2 is independently $—S(R_2)_b—$, $—N(R_2)_b—$ or $—P(R_2)_b—$, wherein b is an integer from 0 to 2;

each R_3 is independently hydrogen or alkyl of 1 to about 10 carbons;

each of R_4 and R_5 is independently a direct bond or alkylene of 1 to about 30 carbons containing 0 to about 15 $—O—$, $—S—$, $—NR_3—$ or $—X_2—(R_3X_2)P(=X_2)—X_2—$ heteroatoms or heteroatom groups; and

each R_6 is independently hydrogen or alkyl of 1 to about 60 carbons containing 0 to about 30 $—O—$, $—S—$, $—NR_3—$ or $—X_2—(R_3X_2)P(=X_2)—X_2—$ heteroatoms or heteroatom groups; with the proviso that the compound of formula (II) comprises at least one, and preferably at least two, quaternary salts.

In the above formula (II), each Y_1 is independently a phosphate residue, $N(R_2)_a—$, $S(R_2)_a—$, $P(R_2)_a—$ or $—CO_2R_2$, wherein a is an integer from 1 to 3. Preferably, each Y_1 is independently a phosphate residue, $—N(R_2)_a—$ or $—CO_2R_2$, wherein a is 2 or 3. Preferably, a is 3.

Also in the above formula (II), R_1 is alkylene of 1 to about 60 carbons containing 0 to about 30 $—O—$, $—S—$, $—NR_3—$ or $—X_2—(R_3X_2)P(=X_2)—X_2—$ heteroatoms or heteroatom groups. Preferably, R_1 is alkylene of 1 to about 40 carbons, with alkylene of 1 to about 20 carbons being preferred. More preferably, R_1 is straight chain alkylene of 1 to about 10 carbons or cycloalkylene of about 4 to about 10 carbons. Even more preferably, R_1 is straight chain alkylene of 1 to about 4 carbons or cycloalkylene of about 5 to about 7 carbons.

In the above definition of Y_1, R_2 is a residue of the formula $—R_4—[(X_1—R_5)_x—Y_2]_y—R_6$, wherein each of x and y is independently an integer from 0 to about 100. Preferably, each of x and y is independently an integer from 0 to about 50, with integers from 0 to about 20 being more preferred. Even more preferably, each of x and y is independently an integer from 0 to about 10.

In the above definition of R_2, each X_1 is independently a direct bond, $—O—$, $—S—$, $—NR_3—$, $—C(=X_2)—$, $—C(=X_2)—N(R_3)—$, $—N(R_3)—C(=X_2)—$, $—C(=X_2)—O—$, $—O—C(=X_2)—$ or $—X_2—(R_3X_2)P(=X_2)—X_2—$. Preferably, X_1 is a direct bond, $—C(=X_2)—N(R_3)—$, $—N(R_3)—C(=X_2)—$, $—C(=X_2)—O—$ or $—O—C(=X_2)—$.

Each X_2 in the above definitions of X_1, R_1, R_4, R_5 and R_6 is independently O or S. Preferably, X_2 is O.

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Each Y_2 in the above definition of R_2 is independently $—S(R_2)_b—$, $—N(R_2)_b—$ or $—P(R_2)_b—$, wherein b is an integer of from 0 to 2. Preferably, Y_2 is $—N(R_2)_b—$ and b is 1 or 2.

In the above definitions of X_1, R_1, R_4, R_5 and R_6, each R_3 is independently hydrogen or alkyl of 1 to about 10 carbons. Preferably, each R_3 is independently hydrogen or alkyl of 1 to about 4 carbons. More preferably, R_3 is hydrogen.

In the above definition of R_2, each of R_4 and R_5 is independently a direct bond or alkylene of 1 to about 30 carbons containing 0 to about 15 $—O—$, $—S—$, $—NR_3—$ or $—X_2—(R_3X_2)P(=X_2)—X_2—$ heteroatoms or heteroatom groups. Preferably, each of R_4 and R_5 is independently a direct bond or alkylene of 1 to about 20 carbons. More preferably, each of R_4 and R_5 is independently a direct bond, straight chain alkylene of 1 to about 10 carbons or cycloalkylene of 4 to about 10 carbons. Even more preferably, each of R_4 and R_5 is independently a direct bond, straight chain alkylene of 1 to about 4 carbons or cycloalkylene of about 5 to about 7 carbons.

Each R_6 in R_2 above is independently hydrogen or alkyl of 1 to about 60 carbons containing 0 to about 30 $—O—$, $—S—$, $—NR_3—$ or $—X_2—(R_3X_2)P(=X_2)—X_2—$ heteroatoms or heteroatom groups. Preferably, each R_6 is independently hydrogen or alkyl of 1 to about 40 carbons. More preferably, each R_6 is independently hydrogen or alkyl of 1 to about 20 carbons.

In yet another embodiment of the present invention, there is provided a cationic lipid compound of the formula

$$\begin{array}{c} (R_4—Y_3)_z \\ | \\ Y_1—(R_1—X_1)_x—R_2—[Y_2—R_3]_y—(X_1—R_1)_x—Y_1 \\ | \qquad\qquad\qquad\qquad\qquad\qquad | \\ (R_1—X_1)_x \qquad\qquad\qquad\qquad (R_1—X_1)_x \\ | \qquad\qquad\qquad\qquad\qquad\qquad | \\ R_2 \qquad\qquad\qquad\qquad\qquad R_2 \\ | \qquad\qquad\qquad\qquad\qquad\qquad | \\ Y_1—(R_1—X_1)_x—[R_3—Y_2]_y—R_2—(X_1—R_1)_x—Y_1 \\ | \\ (R_4—Y_3)_z \end{array} \qquad (III)$$

wherein:

each of x, y and z is independently an integer from 0 to about 100;

each X_1 is independently $—O—$, $—S—$, $—NR_5—$, $—C(=X_2)—$, $—C(=X_2)—N(R_5)—$, $—N(R_5)—C(=X_2)—$, $—C(=X_2)—O—$, $—O—C(=X_2)—$ or $—X_2—(R_5X_2)P(=X_2)—X_2—$;

each X_2 is independently O or S;

each Y_1 is independently $—O—$, $—N(R_6)_a—$, $—S(R_6)_a—$ or $—P(R_6)_a—$, wherein a is an integer from 0 to 2;

each Y_2 is independently $—N(R_6)_a—$, $—S(R_6)_a—$ or $—P(R_6)_a—$, wherein a is an integer from 0 to 2;

each Y_3 is independently a phosphate residue, $N(R_6)_b—$, $S(R_6)_b—$, $P(R_6)_b—$ or $—CO_2R_6$, wherein b is an integer from 1 to 3;

each of R_1, R_2, R_3 and R_4 is independently alkylene of 1 to about 20 carbons;

each R_5 is independently hydrogen or alkyl of 1 to about 10 carbons; and

each R_6 is independently $—[R_7—X_3]_c—R_8$ or $—R_9—[X_4—R_1]_d—Q$, wherein:

each of c and d is independently an integer from 0 to about 100;

each Q is independently a phosphate residue, $—N(R_{11})_q$, $—S(R_{11})_q$, $—P(R_{11})_q$ or $—CO_2R_{11}$, wherein q is an integer from 1 to 3;

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each of X_3 and X_4 is independently —O—, —S—, —NR$_5$—, —C(=X$_2$)—, —C(=X$_2$)—N(R$_5$)—, —N(R$_5$)—C(=X$_2$)—, —C(=X$_2$)—O—, —O—C(=X$_2$)— or —X$_2$—(R$_5$X$_2$)P(=X$_2$)—X$_2$—;

each R_7 is independently alkylene of 1 to about 20 carbons;

each R_8 is independently hydrogen or alkyl of 1 to about 60 carbons;

each of R_9 and R_{10} is independently alkylene of 1 to about 20 carbons; and

each R_{11} is independently —[R$_7$—X$_3$]$_c$—R$_8$ or —R$_9$—[X$_4$—R$_{10}$]$_d$—W, wherein:

 each W is independently a phosphate residue, —N(R$_{12}$)$_w$, —S(R$_{12}$)$_w$, —P(R$_{12}$)$_w$ or —CO$_2$R$_{12}$, wherein w is an integer from 1 to 3; and

 R_{12} is —[R$_7$—X$_3$]$_c$—R$_8$; with the proviso that the compound of formula (III) comprises at least one, and preferably at least two, quaternary salts.

In the above formula (III), each of x, y and z is independently an integer from 0 to about 100. Preferably, each of x, y and z is independently an integer from 0 to about 50, with integers from 0 to about 20 being more preferred. Even more preferably, each of x, y and z is independently an integer from 0 to about 10. Still more preferably, each of x, y and z is independently an integer from 0 to about 5. In certain particularly preferred embodiments, x is 1, y is 2 or 3 and z is 0 or 1.

In the above formula (III), each X_1 is independently —O—, —S—, —NR$_5$—, —C(=X$_2$)—, —C(=X$_2$)—N(R$_5$)—, —N(R$_5$)—C(=X$_2$)—, —C(=X$_2$)—O—, —O—C(=X$_2$)— or —X$_2$—(R$_5$X$_2$)P(=X$_2$)—X$_2$—. Preferably, each X_1 is independently —C(=O)—NR$_5$—, —NR$_5$—C(=O)—, —C(=O)—O— or —O—C(=O)—.

In the above definitions of X_1, X_3 and X_4, each X_2 is independently O or S. Preferably, X_2 is O.

Each Y_1 in formula (III) above is independently —O—, —N(R$_6$)$_a$—, —S(R$_6$)$_a$— or —P(R$_6$)$_a$—, wherein a is an integer from 0 to 2. Preferably, Y_1 is —N(R$_6$)$_a$—, wherein a is 1 or 2.

Each Y_2 in formula (III) above is independently —N(R$_6$)$_a$—, —S(R$_6$)$_a$— or —P(R$_6$)$_a$—, wherein a is an integer from 0 to 2. Preferably, Y_2 is —N(R$_6$)$_a$—.

In the above formula (III), each Y_3 is independently a phosphate residue, N(R$_6$)$_b$—, S(R$_6$)$_b$—, P(R$_6$)$_b$— or —CO$_2$R$_6$, wherein b is an integer from 1 to 3. Preferably, each Y_3 is independently a phosphate residue or N(R$_6$)$_b$—, wherein b is 2 or 3. Preferably, b is 3.

In the above formula (III), each of R_1, R_2, R_3 and R_4 is independently alkylene of 1 to about 20 carbons. Preferably, each of R_1, R_2, R_3 and R_4 is independently straight chain alkylene of 1 to about 10 carbons or cycloalkylene of about 4 to about 10 carbons. More preferably, each of R_1, R_2, R_3 and R_4 is independently straight chain alkylene of 1 to about 4 carbons or cycloalkylene of about 5 to about 7 carbons. Even more preferably, each of R_1, R_2, R_3 and R_4 is independently methylene, ethylene or cyclohexylene.

In the above definitions of X_1, X_3 and X_4, each R_5 is independently hydrogen or alkyl of 1 to about 10 carbons. Preferably, each R_5 is independently hydrogen or alkyl of 1 to about 4 carbons. More preferably, R_5 is hydrogen.

In the above definitions of Y_1, Y_2 and Y_3, each R_6 is independently —[R$_7$—X$_3$]$_c$—R$_8$ or —R$_9$—[X$_4$—R$_{10}$]$_d$—Q, wherein each of c and d is independently an integer from 0 to about 100. Preferably, each of c and d is independently an integer from 0 to about 50, with integers from 0 to about 20 being more preferred. Even more preferably, each of c and d is independently an integer from 0 to about 10, with

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integers from 0 to about 5 being still more preferred. In certain particularly preferred embodiments, c is 0 or 1 and d is 1.

Each Q in R_6 above is independently a phosphate residue, —N(R$_{11}$)$_q$, —S(R$_{11}$)$_q$, —P(R$_{11}$)$_q$ or —CO$_2$R$_{11}$, wherein q is an integer from 1 to 3. Preferably, each Q is independently a phosphate residue, —N(R$_{11}$)$_q$ or —CO$_2$R$_{11}$, wherein q is 2 or 3. Preferably, q is 3.

Also in the above definition of R_6, each of X_3 and X_4 is independently —O—, —S—, —NR$_5$—, —C(=X$_2$)—, —C(=X$_2$)—N(R$_5$)—, —N(R$_5$)—C(=X$_2$), —C(=X$_2$)—O—, —O—C(=X$_2$)— or —X$_2$—(R,X$_2$)P(=X$_2$)—X$_2$—, wherein X_2 and R_5 are as previously described. Preferably, each of X_3 and X_4 is independently —C(=O)—NR$_5$—, —NR$_5$—C(=O)—, —C(=O)—O— or —O—C(=O)—.

In the definitions of R_6, R_{11} and R_{12} above, each R_7 is independently alkylene of 1 to about 20 carbons. Preferably, each R_7 is independently alkylene of 1 to about 10 carbons, with alkylene of 1 to about 4 carbons being preferred. More preferably, each R_7 is independently methylene or ethylene.

Also in the definitions of R_6, R_{11} and R_{12} above, each R_8 is independently hydrogen or alkyl of 1 to about 60 carbons. Preferably, each R_8 is independently hydrogen or alkyl of 1 to about 40 carbons, with hydrogen or alkyl of 1 to about 20 carbons being more preferred. In certain particularly preferred embodiments, each R_8 is independently hydrogen, methyl, dodecyl or hexadecyl.

Each of R_9 and R_{10} in the definitions of R_6 and R_{11} above is independently alkylene of 1 to about 20 carbons. Preferably, each of R_9 and R_{10} is independently alkylene of 1 to about 10 carbons. More preferably, each of R_9 and R_{10} is independently alkylene of 1 to about 4 carbons. Even more preferably, each of R_9 and R_{10} is independently methylene or ethylene.

In Q above, each R_{11} is independently —[R$_7$—X$_3$]$_c$—R$_8$ or —R$_9$—[X$_4$—R$_{10}$]$_d$—W, wherein each of c, d, X_3, X_4, R_7, R_8, R_9 and R_{10} is independently as previously described.

Each W in R_{11} above is independently a phosphate residue, —N(R$_{12}$)$_w$, —S(R$_{12}$)$_w$, —P(R$_{12}$)$_w$ or —CO$_2$R$_{12}$, wherein w is an integer from 1 to 3. Preferably, each W is independently a phosphate residue, —N(R$_{12}$)$_w$ or —CO$_2$R$_{12}$, wherein w is 2 or 3. Preferably, w is 3.

In W above, R_{12} is —[R$_7$—X$_3$]$_c$—R$_8$, wherein each of c, X_3, R_7 and R_8 is independently as previously described.

In the above formulas, it is intended that when any symbol appears more than once in a particular formula or substituent, its meaning in each instance is independent of the other.

Also in the above formulas, it is intended that when each of two or more adjacent symbols is defined as being "a direct bond" to provide multiple, adjacent direct bonds, the multiple and adjacent direct bonds devolve into a single direct bond.

The compounds of formulas (I), (II) and (III) above are exemplary of the cationic lipid compounds which are the subject of the present invention. The cationic or positively charged properties of the cationic lipid compounds is due to the presence of at least one cationic group. In preferred embodiments, at least two cationic groups are present in the cationic lipid compounds of the present invention. The existence of the cationic groups imparts desirable and beneficial properties to the cationic lipid compounds, such properties being absent from lipid compounds known heretofore. In particular, the cationic lipid compounds of the present invention possess improved ability to bind and/or chelate with bioactive agents relative to lipid compounds of the prior art. This binding and/or chelation of the present

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cationic lipid compounds with bioactive agents is referred to generally hereinafter as "interaction". Accordingly, the cationic lipid compounds of the present invention are particularly suitable for use as carriers for bioactive agents and for the intracellular delivery of bioactive agents.

While the inventors do not wish to be bound by any theory or theories of operation, it is believed that the improved ability of the cationic lipid compounds of the present invention to interact with bioactive agents is due, at least in part, to the enhanced charge densities of the present lipid compounds. In this connection, the present cationic lipid compounds possess an increased, positive charge density due to the existence of at least one, and preferably at least two, cationic groups. As discussed in detail below, this enhanced charge density results in unexpectedly desirable interaction with bioactive agents.

Bioactive agents, whether neutral (uncharged) or positively or negatively charged, typically contain a dipole moment and/or one or more heteroatoms, for example, nitrogen, oxygen and sulfur atoms. These heteroatoms generally possess one or more unshared pairs of electrons. It is believed that the positively charged lipid compounds of the present invention electrostatically interact with the negatively charged region of the dipole moment and/or with the unshared pair(s) of electrons on the heteroatoms.

The cationic lipid compounds of the present invention possess particularly improved abilities to interact with bioactive agents which are anionic and which contain one or more anionic groups. Such anionic bioactive agents possess a greater negative charge density relative to neutral or positively charged bioactive agents.

Due to the improved ability of the cationic lipid compounds of the present invention to interact with bioactive agents, the present lipid compounds are particularly suitable for use as carriers for the intracellular delivery of bioactive agents. Thus, the cationic lipid compounds of the present invention are particularly applicable for use in vitro and/or in vivo in methods for the treatment of diseases, including genetic diseases, which involve or require the intracellular delivery of bioactive agents.

As discussed in detail below, the cationic lipid compounds are also particularly suitable for use in the formulation of cationic vesicles, including micelles and liposomes. The inventors have found that cationic liposomes are also particularly suitable for use as carriers for the intracellular delivery of bioactive agents.

As noted above, the cationic lipid compounds of the present invention comprise at least one, and preferably at least two, cationic groups. In an alternate embodiment, the cationic lipid compounds comprise more than at least two cationic groups, for example, at least three cationic groups. In another alternate embodiment, the cationic lipid compounds comprise at least four cationic groups. In yet another alternate embodiment, the cationic lipid compounds comprise at least five cationic groups. In certain embodiments, the cationic lipid compounds comprise more than five cationic groups.

For purposes of illustration only, and not for purposes of limitation, cationic groups may be provided, for example, in the compounds of formula (I) by the group Y_1. Thus, for example, when Y_1 in formula (I) is $N(R_6)_a$— and a is 3, a quaternary salt is formed in that the nitrogen atom of Y_1 is bonded to four other carbon atoms. The nitrogen atom is therefore positively charged.

Other cationic groups, in addition to the cationic groups discussed above, would be apparent to one of ordinary skill in the art based on the present disclosure.

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In embodiments in which the cationic group comprises a quaternary salt, the cationic lipid compound is generally, although not necessarily, associated with a counter ion. Preferably, the counter ion is a pharmaceutically-acceptable counter ion.

In certain preferred embodiments of the present invention, the counter ion is selected from the group consisting of halide, $R_{13}SO_3^-$, $R_{13}CO_2^-$, phosphate, sulfite, nitrate, gluconate, guluronate, galacturonate, estolate and mesylate, wherein R_{13} is hydrogen, alkyl of 1 to about 20 carbons or aryl of about 6 to about 10 carbons. Preferably, R_{13} is hydrogen or alkyl of 1 to about 10 carbons or phenyl. In other preferred embodiments, the counter ion is halide (fluoride, chloride, bromide or iodide), with iodide being preferred. Various other counter ions, including pharmaceutically acceptable counter ions, would be apparent to one skilled in the art based on the present disclosure.

As those skilled in the art will recognize, once placed in possession of the present invention, cationic lipid compositions may be readily formulated from the cationic lipid compounds. Depending on the desired physical properties, cationic lipid compositions may be prepared from the cationic lipid compounds, alone or in combination with other materials, for example, materials which act to stabilize the composition.

It is generally desirable to combine the cationic lipid compounds with other materials, including stabilizing materials, for example, additional amphipathic compounds, to stabilize and/or otherwise improve the properties of the compositions. Compositions which are prepared from the present cationic lipid compounds and additional amphipathic compounds include, for example, suspensions, emulsions, vesicles and hexagonal H II phase structures.

A wide variety of materials which act to stabilize the compositions of the present invention are readily available and would be apparent to a person skilled in the art based on the present disclosure. Included among such materials are additional amphipathic compounds, such as lipids, and fatty materials. The particular stabilizing material which is ultimately combined with the present cationic lipid compounds may be selected as desired to optimize the properties of the resulting composition. It is believed that suitable stabilizing materials are readily identifiable and that compositions of the present cationic lipid compounds can be prepared by one skilled in the art without undue experimentation.

It is also desirable, in certain instances, to combine the cationic lipid compounds with a material which is capable of promoting fusion of the lipid with the cell membrane. Such materials enhance the ability of the cationic lipid compositions to deliver intracellularly the bioactive agent. Certain of such materials are capable also of promoting gene expression. These latter materials are particularly suitable for use in the transfection of genetic material. Examples of materials which are capable of promoting fusion of the cationic lipid composition with cell membranes include, for example, ammonium sulfate, cytochalasin B, chloroquine, glycerol, propylene glycol and poly(ethylene glycol).

In one embodiment of the invention, a cationic lipid composition is provided which comprises a cationic lipid suspension and/or emulsion. Lipid suspensions and emulsions are well known and may be prepared using conventional techniques. As those skilled in the art will recognize, a suspension is a mixture of finely divided particles floating in a liquid, and an emulsion is a colloidal mixture of two or more liquids. The components of the suspension/emulsion are generally mixed together by mechanical agitation, optionally but preferably in the presence of small amounts of additional substances known as emulsifiers.

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Typically, in preparing the suspension/emulsion, the cationic lipid compounds may be added to ethanol or chloroform or any other suitable organic solvent and agitated by hand or by using mechanical techniques. The solvent is then evaporated from the mixture leaving a dried glaze of cationic lipid. The lipids are resuspended in aqueous media, such as phosphate buffered saline, resulting in a suspension/emulsion. To achieve a more homogeneous size distribution of the involved lipids, the mixture may be sonicated using conventional sonication techniques as well as microfluidization (using, for example, a MICROFLUIDIZER™, Newton, Mass.), and/or high pressure extrusion (such as, for example, 600 psi) using an Extruder Device (Lipex Biomembranes, Vancouver, Canada). The lipid may be also subjected to one or more alternating cycles of freezing and thawing to promote the formation of a substantially uniform suspension/emulsion. In addition, a salt, for example, sodium chloride, is optionally added to the suspension/emulsion in a concentration of about 0.05 molar (M) to about 1.0M to promote the formation of substantially uniform dispersions. Bioactive agents may be added to the cationic lipid compounds during the preparation of the suspension/emulsion, such as at the stage where the lipids are added to the organic solvent or at other stages of preparation, or may be added after the cationic lipid suspension/emulsion has been formed, as desired. In preparing the suspensions/emulsions, particularly useful additives are, for example, soybean lecithin, glucose, Pluronic F-68, and D,L-α-tocopherol (Vitamin E), generally in an amount of about 0.03 to about 5 percent by weight. These additives are particularly useful where intravenous applications are desired. Techniques and ingredients for formulating lipid suspensions/emulsions are well known in the art and are applicable to the present cationic suspensions/emulsions. Suitable procedures and suspension/emulsion ingredients are reported, for example, in *Modern Pharmaceutics*, pp. 505–507, Gilbert Baker and Christopher Rhodes, eds., Marcel Dekker Inc., New York, N.Y. (1990), the disclosures of which are hereby incorporated herein by reference in its entirety.

In another embodiment of the invention, a cationic lipid composition is provided which comprises a cationic vesicular composition. The cationic vesicular composition may comprise micelles and/or liposomes. With particular reference to cationic micelle compositions, the following discussion is provided.

Micelles may be prepared using any one of a variety of conventional micellar preparatory methods which will be apparent to those skilled in the art. These methods typically involve suspension of the cationic lipid compound in an organic solvent, evaporation of the solvent, resuspension in an aqueous medium, sonication and centrifugation. The foregoing methods, as well as others, are discussed, for example, in Canfield et al., *Methods in Enzymology*, Vol. 189, pp. 418–422 (1990); El-Gorab et al, *Biochem. Biophys. Acta*, Vol. 306, pp. 58–66 (1973); *Colloidal Surfactant*, Shinoda, K., Nakagana, Tamamushi and Isejura, Academic Press, NY (1963) (especially "The Formation of Micelles", Shinoda, Chapter 1, pp. 1–88); *Catalysis in Micellar and Macro molecular Systems*, Fendler and Fendler, Academic Press, NY (1975). The disclosures of each of the foregoing publications are incorporated by reference herein, in their entirety. The micelles may be prepared in the presence of a bioactive agent or the bioactive agent may be added to pre-formed micelles.

It is generally desirable to include one or more stabilizing materials in the micellar compositions. Exemplary materials

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which may be combined with the cationic lipid compounds to stabilize the micellar compositions produced therefrom include lauryltrimethylammonium bromide, cetyltrimethylammonium bromide, myristyl trimethylammonium bromide, alkyldimethylbenzylammonium chloride, wherein the alkyl group is about 12 to about 16 carbons, benzyldimethyldodecylammonium bromide or chloride, benzyldimethylhexadecylammonium bromide or chloride, benzyldimethyltetradecylammonium bromide or chloride, cetyldimethylethylammonium bromide or chloride, cetylpyridinium bromide and chloride and lauryl sulfate.

Other materials for stabilizing the micellar compositions, in addition to those exemplified above, would be apparent to one skilled in the art based on the present disclosure.

As noted above, the cationic vesicular composition may comprise cationic liposomes. Cationic liposomes are particularly effective as carriers for the intracellular delivery of bioactive agents and are therefore preferred cationic lipid compositions. The present cationic liposomes are highly stable and permit substantially complete entrapment of a bioactive agent within the vesicle. Thus, compositions which comprise cationic liposomes are highly effective carriers for the transfection of bioactive agents in that the liposomes are capable of (A) effectively interacting with the bioactive agent by virtue of electrostatic forces (as discussed above in connection with the cationic lipid compounds, generally); and (B) entrapping the bioactive agent within the liposome vesicle. The cationic liposomes are also highly biocompatible.

The cationic liposome compositions may comprise one or more cationic lipid compounds. In any given liposome, the cationic lipid compound(s) may be in the form of a monolayer or bilayer, and the mono- or bilayer lipids may be used to form one or more mono- or bilayers. In the case of more than one mono- or bilayer, the mono- or bilayers are generally concentric. Thus, the lipids may be used to form a unilamellar liposome (comprised of one monolayer or bilayer), an oligolamellar liposome (comprised of two or three monolayers or bilayers) or a multilamellar liposome (comprised of more than three monolayers or bilayers).

As with the suspensions/emulsions and micelles above, cationic liposome compositions are preferably formulated from both the present cationic lipid compounds and additional stabilizing materials, including additional amphipathic compounds. In the case of liposomes, the additional amphipathic compounds preferably comprise lipids. A wide variety of additional lipids are available which may be incorporated into the liposome compositions. Preferably, the lipids are selected to optimize certain desirable properties of the liposomes, including serum stability and plasma half-life. The selection of suitable lipids in the preparation of cationic liposome compositions would be apparent to a person skilled in the art and can be achieved without undue experimentation, based on the present disclosure.

Lipids which may be used in combination with the resent cationic lipid compounds and in the formulation of cationic liposome compositions include ZONYL™ fluoro surfactants (DuPont Chemicals, Wilmington, Del.) and the fluorine-containing compounds which are described in the following publications: S. Gaentzler et al., *New Journal of Chemistry*, Vol. 17(5), pp. 337–344 (1993); C. Santaella et al., *New Journal of Chemistry*, Vol. 16(3), pp. 399–404 (1992); and L. sole-Violan, *New Journal of Chemistry*, Vol. 17(8,9), pp. 581–583 (1993); the disclosures of each of which are hereby incorporated by reference, in their entireties. Other exemplary lipids which may be used in the preparation of cationic liposome compositions include phos-

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phatidylcholine with both saturated and unsaturated lipids, including dioleoylphosphatidylcholine, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine; phosphatidylethanolamines, such as dioleoylphosphatidylethanolamine and dipalmitoylphosphatidylethanolamine (DPPE); phosphatidylserine; phosphatidylglycerol; sphingolipids; sphingomyelin; lysolipids; glycolipids, such as ganglioside GM1; glucolipids; sulfatides; glycosphingolipids; phosphatidic acids, such as dipalmitoylphosphatidic acid (DPPA); palmitic acid; stearic acid; arachidonic acid; oleic acid; fatty acids; lipids with ether and ester-linked fatty acids; polymerizable lipids; cholesterol, cholesterol sulfate and cholesterol hemisuccinate; 12-{[(7'-diethylaminocoumarin-3-yl)carbonyl]methylamino}octadecanoic acid; N-[12-{[(7'-diethylaminocoumarin-3-yl)carbonyl]methylamino}-octadecanoyl]-2-aminopalmitic acid; cholesteryl-4'-trimethylaminobutanoate; N-succinyldioleoylphosphatidylethanolamine; 1,2-dioleoyl-sn-glycerol; 1,2-dipalmitoyl-sn-3-succinylglycerol; 1,3-dipalmitoyl-2-succinyl-glycerol; 1-hexadecyl-2-palmitoylglycerophosphatidylethanolamine; and palmitoylhomocysteine.

Lipids bearing polymers, including the hydrophilic polymers poly(ethylene glycol) (PEG), polyvinylpyrrolidone, and poly(vinyl alcohol), may also be included in the liposome compositions of the present invention. Examples of suitable hydrophilic polymers include, for example, PEG 2,000, PEG 5,000 and PEG 8,000, which have molecular weights of 2,000, 5,000 and 8,000, respectively. Other suitable polymers, hydrophilic and otherwise, will be readily apparent to those skilled in the art based on the present disclosure. Polymers which may be incorporated via alkylation or acylation reactions onto the surface of the liposome are particularly useful for improving the stability and size distribution of the liposomes. Exemplary lipids which bear hydrophilic polymers include, for example, dipalmitoylphosphatidylethanolamine-PEG, dioleoylphosphatidylethanolamine-PEG and distearylphosphatidyl-ethanolamine-PEG.

Other materials for use in the preparation of cationic liposome compositions, in addition to those exemplified above, would be apparent to one skilled in the art based on the present disclosure.

The amount of stabilizing material, such as, for example, additional amphipathic compound, which is combined with the present cationic lipid compounds may vary depending upon a variety of factors, including the specific cationic lipid compound(s) of the invention selected, the specific stabilizing material(s) selected, the particular use for which it is being employed, the mode of delivery, and the like. The amount of stabilizing material to be combined with the present cationic lipid compounds in a particular situation, and the ratio of stabilizing material to cationic lipid compound, will vary and is readily determinable by one skilled in the art based on the present disclosure. In general, for example, it has been found that higher ratios, that is, ratios higher than about 4:1, 3:1 or 2:1, of cationic lipid compound to stabilizing lipid, are preferred.

A wide variety of methods are available in connection with the preparation of cationic liposome compositions. Accordingly, the cationic liposomes may be prepared using any one of a variety of conventional liposome preparatory techniques which will be apparent to those skilled in the art. These techniques include solvent dialysis, French press, extrusion (with or without freeze thaw), reverse phase evaporation, microemulsification and simple freeze-

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thawing. The liposomes may also be prepared by various processes which involve shaking or vortexing. This may be achieved, for example, by the use of a mechanical shaking device, such as a WIG-L-BUG™ (Crescent Dental, Lyons, Ill.). Conventional microemulsification quipment, such as a MICROFLUIDIZER™ (Microfluidics, Woburn, Mass.) may be used also.

Additional methods for the preparation of liposome ompositions from the cationic lipid compounds of the resent invention include, for example, sonication, chelate dialysis, homogenization, solvent infusion, spontaneous formation, solvent vaporization, controlled detergent dialysis, and others, each involving the preparation of liposomes in various fashions. Methods which involve freeze-thaw techniques are preferred in connection with the preparation of liposomes from the cationic lipid compounds of the present invention. Suitable freeze-thaw techniques are described, for example, in copending U.S. application Ser. No. 07/838,504 now abandoned, filed Feb. 19, 1992, the disclosures of which are incorporated herein by reference in their entirety. Preparation of the liposomes may be carried out in a solution, such as an aqueous saline solution, aqueous phosphate buffer solution, or sterile water, containing one or more bioactive agents, so that the bioactive agent is encapsulated in the liposome or incorporated into the liposome membrane. Alternatively, the bioactive agents may be added to previously formed liposomes.

The size of the liposomes can be adjusted, if desired, by a variety of techniques, including extrusion, filtration, sonication and homogenization. In addition, the size of the liposomes can be adjusted by the introduction of a laminar stream of a core of liquid into an immiscible sheath of liquid. Other methods for adjusting the size of the cationic liposomes and for modulating the resultant liposomal biodistribution and clearance of the liposomes would be apparent to one skilled in the art based on the present disclosure. Preferably, the size of the cationic liposomes is adjusted by extrusion under pressure through pores of a defined size. Although liposomes employed in the subject invention may be of any one of a variety of sizes, preferably the liposomes are small, that is, less than about 100 nanometer (nm) in outside diameter.

Many of the foregoing liposomal preparatory techniques, as well as others, are discussed, for example, in U.S. Pat. No. 4,728,578; U.K. Patent Application GB 2193095 A; U.S. Pat. No. 4,728,575; U.S. Pat. No. 4,737,323; International Application Serial No. PCT/US85/01161; Mayer et al., *Biochimica et Biophysica Acta*, Vol. 858, pp. 161–168 (1986); Hope et al., *Biochimica et Biophysica Acta*, Vol. 812, pp. 55–65 (1985); U.S. Pat. No. 4,533,254; Mayhew et al., *Methods in Enzymology*, Vol. 149, pp. 64–77 (1987); Mayhew et al., *Biochimica et Biophysica Acta*, Vol 755, pp. 169–74 (1984); Cheng et al, *Investigative Radiology*, Vol. 22, pp. 47–55 (1987); International Application Serial No. PCT/US89/05040; U.S. Pat. No. 4,162,282; U.S. Pat. No. 4,310,505; U.S. Pat. No. 4,921,706; and *Liposome Technology*, Gregoriadis, G., ed., Vol. I, pp. 29–31, 51–67 and 79–108 (CRC Press Inc., Boca Raton, Fla. 1984), the disclosures of each of which are hereby incorporated by reference herein, in their entirety.

Although any of a number of varying techniques can be used, the liposomes of the present invention are preferably prepared using a shaking technique. Preferably, the shaking techniques involve agitation with a mechanical shaking apparatus, such as a WIG-L-BUG™ (Crescent Dental, Lyons, Ill.), such as those disclosed in copending U.S. application Ser. No. 160,232, filed Nov. 30, 1993, (issued as

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U.S. Pat. No. 5,542,935), the disclosures of which are hereby incorporated herein by reference in their entirety.

As those skilled in the art will recognize, any of the cationic lipid compounds and compositions containing the cationic lipid compounds, with or without bioactive agents, may be lyophilized for storage, and reconstituted in, for example, an aqueous medium (such as sterile water or phosphate buffered solution, or aqueous saline solution), with the aid of vigorous agitation. To prevent agglutination or fusion of the lipids as a result of lyophilization, it may be useful to include additives which prevent such fusion or agglutination from occurring. Additives which may be useful include sorbitol, mannitol, sodium chloride, glucose, trehalose, polyvinylpyrrolidone and poly(ethylene glycol), for example, PEG 400. These and other additives are described in the literature, such as in the U.S. Pharmacopeia, USP XXII, NF XVII, The United States Pharmacopeia, The National Formulary, United States Pharmacopeial Convention Inc., 12601 Twinbrook Parkway, Rockville, Md. 20852, the disclosures of which are hereby incorporated herein by reference in their entirety. Lyophilized preparations generally have the advantage of greater shelf life.

The inventors have found that intracellular delivery of bioactive agents through the use of cationic lipid compositions, including suspensions/emulsions and vesicular compositions, may be enhanced by the presence of a gaseous substance. It is contemplated that the gaseous substance promotes uptake by cells of the bioactive agent. Thus, in certain preferred embodiments, a gas, such as an inert gas, is incorporated in the cationic lipid compositions. Alternatively, a precursor to a gaseous substance may be incorporated in the cationic lipid compositions. Such precursors include, for example, materials which are capable of converting in vivo to a gas, and preferably, to an inert gas.

Preferred gases are gases which are inert and which are biocompatible, that is, gases which are not injurious to biological function. Preferable gases include those selected from the group consisting of air, noble gases, such as helium, neon, argon and xenon, carbon dioxide, nitrogen, fluorine, oxygen, sulfur hexafluoride, fluorocarbons, perfluorocarbons, and mixtures thereof. Other gases, including the gases exemplified above, would be readily apparent to one skilled in the art based on the present disclosure.

In preferred embodiments, the gas comprises a perfluorocarbon. Preferably, the perfluorocarbon is selected from the group consisting of perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluorocyclobutane, and mixtures thereof. More preferably, the perfluorocarbon gas is perfluoropropane or perfluorobutane, with perfluoropropane being particularly preferred.

As noted above, it may also be desirable to incorporate in the cationic lipid compositions a precursor to a gaseous substance. Such precursors include materials that are capable of being converted in vivo to a gas. Preferably, the gaseous precursor is biocompatible, and the gas produced in vivo is biocompatible also.

Among the gaseous precursors which are suitable for use in the present compositions are pH sensitive agents. These agents include materials that are capable of evolving gas, for example, upon being exposed to a pH that is neutral or acidic. Examples of such pH sensitive agents include salts of an acid which is selected from the group consisting of inorganic acids, organic acids and mixtures thereof. Carbonic acid (H_2CO_3) is an example of a suitable inorganic acid, and aminomalonic acid is an example of a suitable organic acid. Other acids, including inorganic and organic acids, would be readily apparent to one skilled in the art based on the present disclosure.

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Preferably, the gaseous precursor is a salt which is selected from the group consisting of an alkali metal salt, an ammonium salt and mixtures thereof. More preferably, the salt is selected from the group consisting of carbonate, bicarbonate, sesquecarbonate, aminomalonate and mixtures thereof.

Examples of gaseous precursor materials for use in the cationic lipid compositions of the present invention include lithium carbonate, sodium carbonate, potassium carbonate, lithium bicarbonate, sodium bicarbonate, potassium bicarbonate, magnesium carbonate, calcium carbonate, magnesium bicarbonate, ammonium carbonate, ammonium bicarbonate, ammonium sesquecarbonate, sodium sesquecarbonate, sodium aminomalonate and ammonium aminomalonate. Aminomalonate is well known in the art, and its preparation is described, for example, in Thanassi, *Biochemistry*, Vol. 9, no. 3, pp. 525–532 (1970); Fitzpatrick et al., *Inorganic Chemistry*, Vol. 13, no. 3 pp. 568–574 (1974); and Stelmashok et al., *Koordinatsionnaya Khimiya*, Vol. 3, no. 4, pp. 524–527 (1977). The disclosures of these publications are hereby incorporated herein by reference.

In addition to, or instead of, being sensitive to changes in pH, the gaseous precursor materials may also comprise compounds which are sensitive to changes in temperature. Such temperature sensitive agents include materials which have a boiling point of greater than about 37° C. Exemplary temperature sensitive agents are methyl lactate, perfluoropentane and perfluorohexane. The gaseous precursor materials may be also photoactivated materials, such as diazonium ion and aminomalonate. As discussed more fully hereinafter, certain lipid compositions, and particularly vesicular compositions, may be designed so that gas is formed at the target tissue or by the action of sound on the particle. Examples of gaseous precursors are described, for example, in U.S. Pat. Nos. 5,088,499 and 5,149,319. These patents are hereby incorporated herein by reference in their entirety. Other gaseous precursors, in addition to those exemplified above, will be apparent to one skilled in the art based on the present disclosure.

In certain preferred embodiments, a gaseous agent, for example, air or a perfluorocarbon gas, is combined with a liquid perfluorocarbon, such as perfluorohexane, perfluoroheptane, perfluorooctylbromide (PFOB), perfluorodecalin, perfluorododecalin, perfluorooctyliodide, perfluorotripropylamine and perfluorotributylamine.

A preferred composition for use in the intracellular delivery of a bioactive agent, for example, genetic material, comprises a bioactive agent, a perfluorocarbon gas and a gaseous precursor which has a boiling point of greater than about 37° C., such as perfluoropentane. As discussed in detail below, energy, for example, heat or ultrasound, is preferably applied to the patient after the administration of the composition and to assist in the intracellular delivery of the bioactive agent.

The gaseous substances and/or gaseous precursors are preferably incorporated in the cationic lipid compositions of the present invention irrespective of the physical nature of the composition. Thus, it is contemplated that the gaseous substances and/or precursors thereto are incorporated in compositions which are suspensions/emulsions or vesicular compositions, including micelles and liposomes. Incorporation of the gaseous substances and/or precursors thereto in the cationic lipid compositions may be achieved by using any of a number of methods. For example, the formation of gas-filled vesicles can be achieved by shaking or otherwise agitating an aqueous mixture which comprises a gas or gas precursor and the cationic lipids of the present invention.

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This promotes the formation of stabilized vesicles within which the gas or gas precursor is encapsulated. Gas or gaseous precursor cationic lipid compositions may be prepared in other manners similar to those discussed in connection with the incorporation of bioactive agents in vesicular compositions as earlier discussed.

The gaseous substances and/or precursors thereto may also be incorporated in the cationic lipid compositions using any conventional and well-known techniques. For example, a gas may be bubbled directly into an aqueous mixture of the present cationic lipid compounds, optionally in the presence of a bioactive agent. Alternatively, a gas instillation method can be used as disclosed, for example, in U.S. Pat. Nos. 5,352,435 and 5,228,446, the disclosures of each of which are hereby incorporated herein by reference in their entireties. Suitable methods for incorporating the gas or gas precursor in cationic lipid compositions are disclosed also in U.S. Pat. No. 4,865,836, the disclosure of which is hereby incorporated herein by reference. Other methods would be apparent to one skilled in the art based on the present disclosure.

In preferred embodiments, the gaseous substances and/or gaseous precursor materials are incorporated in vesicular compositions, with micelles and liposomes being preferred. Liposomes are particularly preferred because of their high stability and biocompatability. As discussed in detail below, vesicles in which a gas or gas precursor or both are encapsulated are advantageous in that they can be more easily monitored in vivo, for example, by monitoring techniques which involve ultrasound. Thus, the circulation and delivery of the vesicles to the targeted tissue and/or cells can be observed via a non-invasive procedure. Gas precursor- or gas-filled vesicles are preferred also because the application of high energy ultrasound, radio frequency, optical energy, for example, laser light, and/or heat, to produce areas of hyperthermia, can be used to rupture in vivo the vesicles and thereby promote release of the entrapped gas (or precursor thereto) and bioactive agent. Thus, vesicular compositions permit the controlled release of a bioactive agent in vivo.

In addition to being entrapped within the vesicle, it is contemplated that the bioactive agent may be located also, or instead of, outside of the vesicles or in the lipid membranes. Thus, in certain embodiments, the bioactive agent may be coated on the surface of the liposomes or micelles and/or in the lipid membranes, in addition to, or instead of, being entrapped within the vesicles.

The bioactive agent which is incorporated in the present cationic lipid compositions is preferably a substance which is capable of exerting a therapeutic biological effect in vitro and/or in vivo. Particularly suitable bioactive agents for use in the methods and compositions of the present invention is genetic material. Examples of genetic materials include, for example, genes carried on expression vectors, such as plasmids, phagemids, cosmids, yeast artificial chromosomes (YACs) and defective- or "helper" viruses; anti-sense and sense oligonucleotides; phosphorothioate oligodeoxynucleotides; antigene nucleic acids; and single and double stranded RNA and DNA, including DNA which encodes at least a portion of a gene, for example, DNA which encodes for human leukocyte antigen (HLA), dystrophin, cystic fibrosis transmembrane receptor (CFTR), interleukin-2 (IL-2), tumor necrosis factor (TNF) and granulocyte-macrophage colony stimulating factor (GMCSF). The DNA can also encode certain proteins which may be used in the treatment of various types of pathologies or conditions, including those which are associated with the loss or deterioration of immune competence. Such pathologies or con-

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ditions involving immune competence include, for example, acquired immune deficiency syndrome (AIDS), cancer, chronic viral infections, and autoimmune disease.

Specifically, DNA may be selected which expresses adenosine deaminase (ADA) for the treatment of ADA deficiency; growth hormone for the treatment of growth deficiency or to aid in the healing of tissues; insulin for the treatment of diabetes; luteinizing hormone releasing hormone (LHRH) antagonist as a birth control agent; LHRH for the treatment of prostate or breast cancer; tumor necrosis factor and/or interleukin-2 for the treatment of advanced cancers; high-density lipoprotein (HDL) receptor for the treatment of liver disease; thymidine kinase for the treatment of ovarian cancer, brain tumors, or human immunodeficiency virus (HIV) infection; HLA-B7 for the treatment of malignant melanoma; IL-2 for the treatment of neuroblastoma, malignant melanoma or kidney cancer; interleukin-4 (IL-4) for the treatment of cancer; HIV env for the treatment of HIV infection; antisense ras/p53 for the treatment of lung cancer; and Factor VIII for the treatment of Hemophilia B. Such therapies are described, for example, in *Science*, Vol. 258, pp. 744–746 (1992), the disclosure of which is incorporated herein by reference in its entirety.

As noted above, the present invention provides cationic lipid formulations which comprise cationic lipid compositions in combination with one or more bioactive agents. The cationic lipid compositions may comprise cationic suspensions/emulsions and/or cationic vesicular compositions, including cationic liposome compositions and/or cationic micelle compositions. In addition, the cationic lipid compositions can comprise one or more cationic lipid compounds optionally in combination with a stabilizing material, such as an amphipathic compound, and a gas or precursor thereto. These cationic lipid formulations may be prepared according to any of a variety of techniques. For example, the cationic lipid formulations may be prepared from a mixture of cationic lipid compounds, bioactive agent and gas or gaseous precursor. In the case of vesicular compositions, it is contemplated that the bioactive agent is entrapped within the vesicle of the liposome or micelles. In certain cases, the bioactive agent can be incorporated also into the membrane walls of the vesicle. In the case of a suspension/emulsion, it is contemplated that the bioactive agent is generally dispersed homogeneously throughout the suspension/emulsion. Alternatively, the cationic lipid compositions may be preformed from cationic lipid compounds and gas or gaseous precursor. In the latter case, the bioactive agent is then added to the lipid composition prior to use. For example, an aqueous mixture of liposomes and gas may be prepared to which the bioactive agent is added and which is agitated to provide the cationic liposome formulation. The cationic liposome formulation is readily isolated also in that the gas- and/or bioactive agent-filled liposome vesicle generally float to the top of the aqueous solution. Excess bioactive agent can be recovered from the remaining aqueous solution.

The formulations of the present invention can be used in either in vitro or in vivo applications. In the case of in vitro applications, including cell culture applications, the cationic lipid formulations can be added to the cells in cultures and then incubated. If desired, where liposomes are employed, energy, such as sonic energy, may be applied to the culture media to burst the liposomes and release any therapeutic agents.

With respect to in vivo applications, the formulations of the present invention can be administered to a patient in a variety of forms adapted to the chosen route of

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administration, namely, parenterally, orally, or intraperitoneally. Parenteral administration, which is preferred, includes administration by the following routes: intravenous; intramuscular; interstitially; intraarterially; subcutaneous; intra ocular; intrasynovial; trans epithelial, including transdermal; pulmonary via inhalation; ophthalmic; sublingual and buccal; topically, including ophthalmic; dermal; ocular; rectal; and nasal inhalation via insufflation. Intravenous administration is preferred among the routes of parenteral administration.

It is contemplated that the present cationic lipid formulations can be administered also by coating a medical device, for example, a catheter, such as an angioplasty balloon catheter, with a cationic lipid formulation. Coating may be achieved, for example, by dipping the medical device into a cationic lipid formulation or a mixture of a cationic lipid formulation and a suitable solvent, for example, an aqueous-based buffer, an aqueous solvent, ethanol, methylene chloride, chloroform and the like. An amount of the formulation will naturally adhere to the surface of the device which is subsequently administered to a patient, as appropriate. Alternatively, a lyophilized mixture of a cationic lipid formulation may be specifically bound to the surface of the device. Such binding techniques are described, for example, in K. Ishihara et al., *Journal of Biomedical Materials Research*, Vol. 27, pp. 1309–1314 (1993), the disclosures of which are incorporated herein by reference in their entirety.

The useful dosage to be administered and the particular mode of administration will vary depending upon such factors as the age, weight and the particular animal and region thereof to be treated, the particular bioactive agent and cationic lipid compound used, the therapeutic or diagnostic use contemplated, and the form of the formulation, for example, suspension, emulsion, micelle or liposome, as will be readily apparent to those skilled in the art. Typically, dosage is administered at lower levels and increased until the desirable therapeutic effect is achieved. The amount of cationic lipid compound that is administered can vary and generally depends upon the amount of bioactive agent being administered. For example, the weight ratio of cationic lipid compound to bioactive agent is preferably from about 1:1 to about 15:1, with a weight ratio of about 5:1 to about 10:1 being more preferred. Generally, the amount of cationic lipid compound which is administered will vary from between about 0.1 milligram (mg) to about 1 gram (g). By way of general guidance, typically between about 0.1 mg and about 10 mg of the particular bioactive agent, and about 1 mg to about 100 mg of the cationic lipid compositions, each per kilogram of patient body weight, is administered, although higher and lower amounts can be used.

After vesicular lipid formulations which comprise a gas or gaseous precursor and bioactive agent have been administered to a patient, energy, preferably in the form of ultrasonic energy, can be applied to the target tissue to identify the location of the vesicles containing gas or gaseous precursor and bioactive agent. The applied energy may also be employed to effect release of the bioactive agent and facilitates cellular uptake of the bioactive agent. As one skilled in the art would recognize, this method of mediating cellular transfection with ultrasonic energy is preferably effected with tissues whose acoustic window permits the transmission of ultrasonic energy. This is the case for most tissues in the body, including muscle and organ tissues, such as the heart and liver, as well as most other vital structures. With respect to brain tissue, it may be necessary to create a "surgical window" by removing part of the skull, inasmuch as ultrasonic energy generally does not transmit through

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bone. Intravascular and/or endoluminal ultrasound transducers may be used to apply the ultrasound energy to selected tissues and/or sites in the body, for example, the aorta and the esophagus.

Cationic lipid formulations can be formulated to be sufficiently stable in the vasculature such that they circulate throughout the body and provide blood pool equilibration. As one skilled in the art would recognize, the lipid formulations, including those which comprise suspensions/emulsions and vesicles, such as liposomes and micelles, may be coated with certain materials to minimize uptake by the reticuloendothelial system. Suitable coatings include, for example, gangliosides and glycolipids which bind saccharide moieties, such as glucuronate, galacturonate, guluronate, poly(ethylene glycol), poly(propylene glycol), polyvinylpyrrolidone, poly(vinyl alcohol), dextran, starch, phosphorylated and sulfonated mono-, di-, tri-, oligo- and polysaccharides and albumin. Provided that the circulation half-life of the cationic lipid formulations is of a sufficient period of time, they will generally pass through the target tissue while passing through the body. In the case of lipid formulations which comprise gas or gaseous precursors, energy, for example, sonic energy, may be focused on the tissue to be treated, for example, diseased tissue. The bioactive agent will then be released locally in the target tissue. The inventors have found also that antibodies, carbohydrates, peptides, glycopeptides, glycolipids and lectins also assist in the targeting of tissue with the lipid formulations and the bioactive agents. Accordingly, these materials may be incorporated into the lipid formulations also.

Ultrasound can be used for both diagnostic and therapeutic purposes. In general, the levels of energy from diagnostic ultrasound are insufficient to cause rupture of vesicular species and to facilitate release and cellular uptake of the bioactive agents. Moreover, diagnostic ultrasound involves the application of one or more pulses of sound. Pauses between pulses permits the reflected sonic signals to be received and analyzed. The limited number of pulses used in diagnostic ultrasound limits the effective energy which is delivered to the tissue that is being studied.

On the other hand, higher energy ultrasound, for example, ultrasound which is generated by therapeutic ultrasound equipment, is generally capable of causing rupture of the vesicular species. In general, therapeutic ultrasound machines use from about 10 to about 100% duty cycles, depending on the area of tissue to be treated with the ultrasound. Areas of the body which are generally characterized by larger amounts of muscle mass, for example, backs and thighs, as well as highly vascularized tissues, such as heart tissue, may require a larger duty cycle, for example, up to about 100%.

In therapeutic ultrasound, continuous wave ultrasound is used to deliver higher energy levels. For the rupture of vesicular species, continuous wave ultrasound is preferred, although the sound energy may be pulsed also. If pulsed sound energy is used, the sound will generally be pulsed in echo train lengths of about 8 to about 20 or more pulses at a time. Preferably, the echo train lengths are about 20 pulses at a time. In addition, the frequency of the sound used may vary from about 0.25 to about 100 megahertz (MHz). In general, frequency for therapeutic ultrasound ranges between about 0.75 and about 3 MHz are preferred with about 1 and about 2 MHz being more preferred. In addition, energy levels may vary from about 0.05 Watt (W) to about 5.0 W, with energy levels of about 0.1 to about 0.5 W being preferred. For very small vesicular species, for example,

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species in which the vesicles have a diameter of less than about 0.5 micron, higher frequencies of sound are generally preferred. This is because smaller vesicular species are capable of absorbing sonic energy more effectively at higher frequencies of sound. When very high frequencies are used, for example, greater than about 10 MHz, the sonic energy will generally penetrate fluids and tissues to a limited depth only. Thus, external application of the sonic energy may be suitable for skin and other superficial tissues. However, for deep structures it is generally necessary to focus the ultrasonic energy so that it is preferentially directed within a focal zone. Alternatively, the ultrasonic energy may be applied via interstitial probes, intravascular ultrasound catheters or endoluminal catheters. Such probes or catheters may be used, for example, in the esophagus for the diagnosis and/or treatment of esophageal carcinoma.

The present invention is further described in the following examples. In these examples, examples 1 to 9 are actual examples. Examples 10 to 12 are prophetic examples. These examples are for illustrative purposes only, and are not to be construed as limiting the appended claims.

Various of the starting materials used in the following examples are commercially available. N,N-dimethylethylenediamine and iodomethane were purchased from Aldrich Chemical Co. (Milwaukee, Wis.).

EXAMPLE 1

Synthesis of N,N'-Bis (dodecylaminocarbonylmethylene)-N,N-'-bis (β-N,N, N-trimethylammoniumethylaminocarbonylmethylene)-N,N'-dimethylethylenediamine tetraiodide (EDTA-LA-TMA tetraiodide)
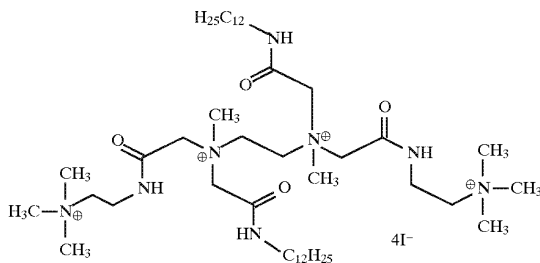
Synthetic Route

(i) Synthesis of N,N'-Bis (dodecylaminocarbonylmethylene)-ethylenediamine-N,N'-diacetic acid (EDTA-LA)
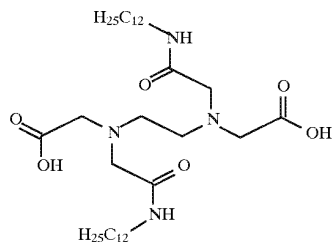
Dodecylamine (3.71 g, 0.02 mole) in dry methanol (60 mL) was added to a suspension of ethylenediaminetetraace-

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tic acid dianhydride (2.56 g, 0.01 mole) in dry methanol (30 mL). The mixture was stirred at 50° C. for 6 hours. The resulting white solid precipitate was isolated by filtration and dried under vacuum at room temperature to yield 3.43 g (64%) of the title compound. m.p. 156°–158° C.

IR: 3320 cm^{-1} for OH; 1670 cm^{-1} for —C(═O)—.

(ii) Synthesis of N,N'-Bis (dodecylaminocarbonylmethylene)-N,N'-bis(β-N,N-dimethylaminoethylaminocarbonylmethylene) ethylenediamine (EDTA-LA-DMA)
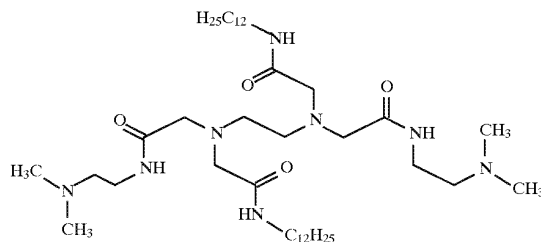
EDTA-LA (3.14 g, 0.005 mole) from step (i), N,N-dimethylethylenediamine (0.88 g, 0.01 mole) and CHCl$_3$ (100 mL) were combined. After dissolution of the solid materials, the solution was cooled to 50° C. and a solution of 1,3-dicyclohexylcarbodiimide (DCC) (2.227 g, 0.011 mole) in CHCl$_3$ (20 mL) was added dropwise. A precipitate was observed. The reaction mixture was stirred at room temperature for about 24 hours. The reaction mixture was filtered and the filtrate was washed with 0.59 acetic acid (100 mL) to decompose any excess DCC. A white milky solution was formed which separated into two layers. The bottom organic layer was dried (Na$_2$SO$_4$) and concentrated in vacuo to yield 3.81 g of the title compound as a soft solid.

IR: 3280 cm-1; 2900 cm^{-1}; 1640 cm^{-1}; 1530 cm^{-1}.

(iii) Synthesis of EDTA-LA-TMA Tetraiodide

A solution of EDTA-LA-DMA (3.66 g, 4.77 mmole) from step (ii), iodomethane (3.41 g, 24 mmole) and ethanol (30 mL) was refluxed for 2 hours. The ethanolic solution was concentrated in vacuo and the resulting residue was lyophilized overnight. 3.98 g of EDTA-LA-TMA tetraiodide, a compound within the scope of the invention, was obtained as a yellow solid.

IR: 3260 cm^{-1}; 1650 cm^{-1}.

EXAMPLE 2

Synthesis of N,N"-Bis (hexadecylaminocarbonylmethylene)-N,N'N"-tris(β-N,N, N-trimethylammoniumethylaminocarbonylmethylene)-N,N',N"-trimethyldiethylenetriamine hexaiodide (DTPA-HA-TME Hexaiodide)

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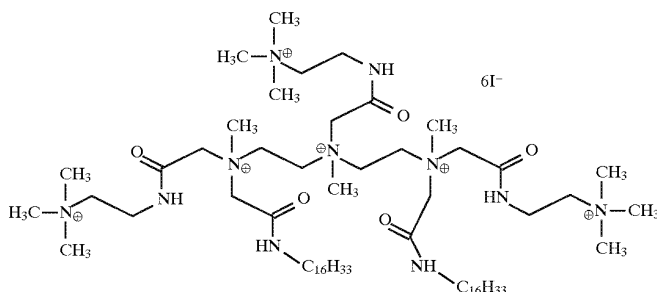
Synthetic Route

(i) Synthesis of N,N"-Bis
(hexadecylaminocarbonylmethylene)-
diethylenetriamine-N,N',N"-triacetic acid
(DTPA-HA)
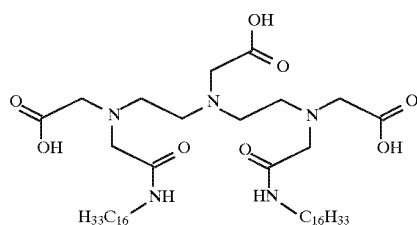
Hexadecylamine (4.82 g, 0.02 mole) in dry methanol(60 mL) was added to a suspension of diethylenetriamine pentaacetic acid dianhydride (3.57 g, 0.01 mole) in dry methanol (30 mL). The resulting mixture was stirred at 50° C. for 6 hours. The reaction mixture was cooled and the resulting white solid precipitate was collected by filtration. The white solid was dried under vacuum to yield 5.9 g of the title compound.

(ii) Synthesis of N,N"-Bis
(hexadecylaminocarbonylmethylene)-N,N',N"-tris(β-
N,N-dimethylaminoethylaminocarbonylmethylene)
diethylenetriamine (DTPA-HA-DMA)
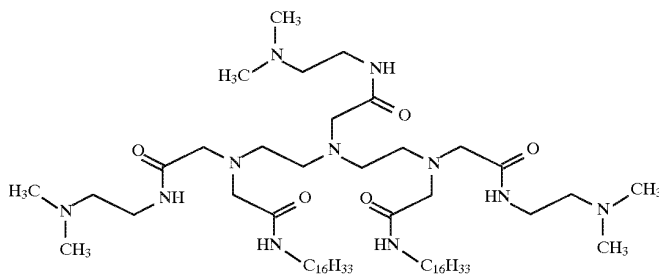
A solution of DTPA-HA (4.2 g, 0.005 mole) from step (i), N,N-dimethylethylenediamine (0.88 g, 0.01 mole) and CHCl$_3$ (100 mL) was cooled to 0°–5° C. To this solution was added dropwise a solution of DCC (2.23 g, 0.011 mole) in CHCl$_3$ (20 mL). The reaction mixture was stirred for 24 hours at room temperature. The resulting precipitate was removed by filtration and was washed with 0.5% acetic acid (100 mL). A white, milky solution was obtained which was filtered again, dried (Na$_2$SO$_4$), and concentrated in vacuo. The title compound was obtained as a soft solid (3.5 g).

(iii) Synthesis of DTPA-HA-TMA Hexaiodide

A solution of DTPA-HA-DMA (4.7 g, 4.8 mmole) from step (ii), iodomethane (3.41 g) and methanol (50 mL) was refluxed for 2 hours. The methanolic solution was concentrated in vacuo and the resulting residue was lyophilized overnight. 6 g of DTPA-HA-TME hexaiodide, a compound within the scope of the invention, was obtained as a yellow solid.

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EXAMPLE 3

Synthesis of N,N'-Bis
(dodecylaminocarbonylmethylene)-N,N'-bis(β-N,N,
N-
trimethylammoniumethylaminocarbonylmethylene)-
N, N'-dimethyl cyclohexylene-1,4-diamine
tetraiodide (CDTA-LA-TMA Tetraiodide)
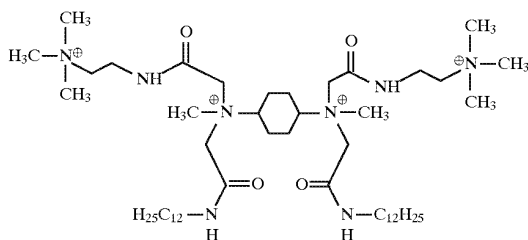
Synthetic Route

(i) Synthesis of N,N'-Bis
(dodecylaminocarbonylmethylene)-cyclohexylene-1,
4-diamine-N,N'-diacetic acid (CDTA-LA)
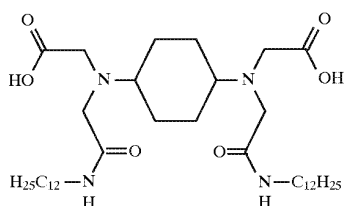
A solution of dodecylamine (3.71 g, 0.02 mole) in dry
methanol (60 ml) was added to a suspension of cyclohexane-

A solution of CDTA-LA (3.4 g, 0.005 mole) from step (i),
N,N-dimethylethylenediamine (0.88 g, 0.01 mole) and
$CHCl_3$ (100 mL) was cooled to 0°–5° C. To this solution was
added dropwise a solution of DCC (2.23 g, 0.011 mole) in
$CHCl_3$ (20 mL). The reaction mixture was stirred for 24
hours at room temperature and filtered. The filtrate was
washed with 0.5% acetic acid (100 mL) to decompose any
excess DCC and was filtered again. The filtrate was dried
(Na_2SO_4) and concentrated in vacuo to yield the title com-
pound (4.2 g) as a soft solid.

(iii) Synthesis of CDTA-LA-TMA Tetraiodide

A solution of CDTA-LA-DMA (3 g) from step (ii),
iodomethane (3.5 g) and methanol (30 mL) was refluxed for
2 hours. The methanolic mixture was concentrated in vacuo
and the resulting residue was lyophilized overnight. 3.1 g of
CDTA-LA-TMA tetraiodide, a compound within the scope
of the invention, was obtained as a solid.

EXAMPLE 4

Synthesis of 1,1,7,7-tetra(β-N,N,N,N-
tetramethylammoniumethylaminocarbonylmethylene)-
4-hexadecylaminocarbonylmethylene-N,N',N"-
trimethyl-1,4,7-triazaheptane heptaiodide (DTPA-
MHA-TTMA Heptaiodide)

1,4-diamine-N,N,N',N'-tetraacetic acid dianhydride (3.1 g,
0.01 mole) in dry methanol (30 mL). The resulting mixture
was stirred at 50° C. for 6 hours. Filtration yielded the title
compound (4.8 g) as a white solid.

(ii) Synthesis of N,N'-Bis
(dodecylaminocarbonylmethylene)-N,N'-bis(β-N,N-
dimethylaminoethylaminocarbonyl methylene)
cyclohexylene-1,4-diamine (CDTA-LA-DMA)
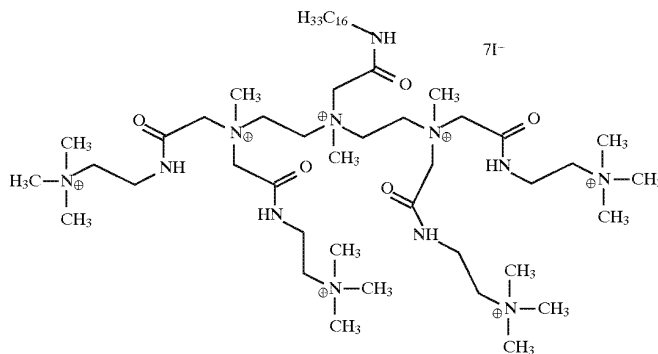
Synthetic Route

(i) Synthesis of 4-
hexadecylaminocarbonylmethylene-1,4,7-
triazaheptane-1,1,7,7-tetraacetic acid (DTPA-MHA)

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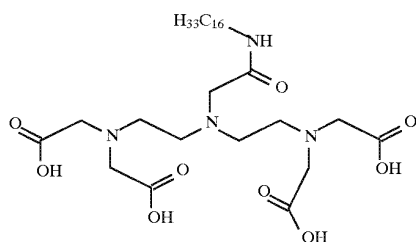
A solution of diethylenetriaminepentaacetic acid (3.93 g, 0.01 mole), hexadecylamine (2.4 g, 0.01 mole) and CHCl$_3$ (200 mL) was cooled to 0°–50° C. To this solution was added dropwise a solution of DCC (2.23 g, 0.011 mole) in CHCl$_3$ (15 mL). The reaction mixture was stirred for 24 hours at room temperature and filtered. The filtrate was washed with 0.5% acetic acid (100 mL) and filtered again. The filtrate was dried (Na$_2$SO$_4$) and concentrated in vacuo. Recrystallization of the resulting residue from water yielded the title compound as a white solid (3.7 g).

(ii) Synthesis of 1,1,7,7-tetra(β-N,N-dimethylaminoethylaminocarbonylmethylene)-4-hexadecylaminocarbonylmethylene)-1,4,7-triazaheptane (DTPA-MHA-TDMA)
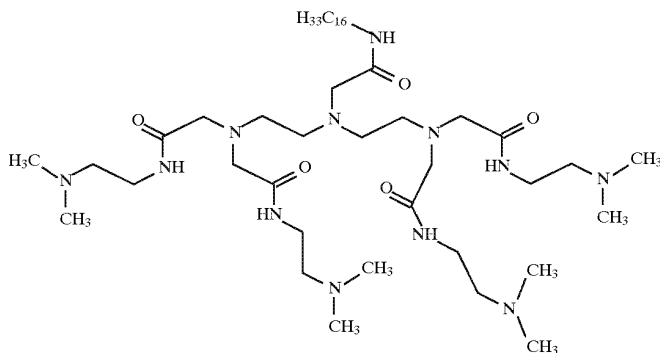
A solution of DTPA-MHA (3 g) from step (i), N,N-dimethylethylenediamine (1.76 g) and CHCl$_3$ (200 mL) was cooled to 0°–5° C. To this solution was added dropwise a solution of DCC (4.5 g, 0.02 mole) in CHCl$_3$ (20 mL). The reaction mixture was stirred overnight at room temperature. The resulting precipitate was collected by filtration and the filtrate was washed with 0.5% acetic acid (100 mL) and filtered again. The filtrate was dried (Na$_2$SO$_4$) and concentrated in vacuo. The resulting residue was purified on a silica gel column. The title compound was obtained as a soft solid (2.8 g).
(iii) Synthesis of DTPA-MHA-TTMA Heptaiodide
A solution of DTPA-MHA-TDMA (2.24 g), iodomethane (3.5 g, 0.03 mole) and methanol (30 mL) was refluxed for 2

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hours. The methanolic solution was concentrated in vacuo and the resulting residue was lyophilized overnight to yield 2.4 g of DTPA-MHA-TTMA heptaiodide, a compound within the scope of the present invention.

EXAMPLE 5

Using procedures similar to those in Examples 1 to 4, the following compounds within the scope of the invention were prepared.

EXAMPLE 5A

N,N'-Bis(dodecyloxycarbonylmethylene)-N,N'-bis(β-N,N, N-trimethylammoniumethylaminocarbonylmethylene) ethylenediamine diiodide
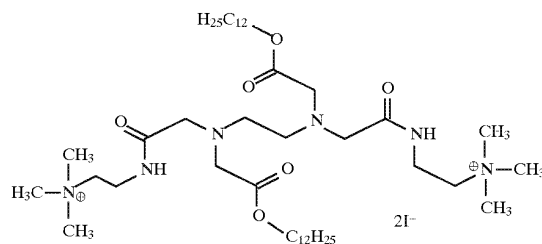
EXAMPLE 5B

N,N,N",N"-Tetra(β-N,N,N-trimethylammoniumethylaminocarbonylmethylene)-N'-(1,2-dioleoylglycero-3-phosphoethanolaminocarbonylmethylene) diethylenetriamine tetraiodide

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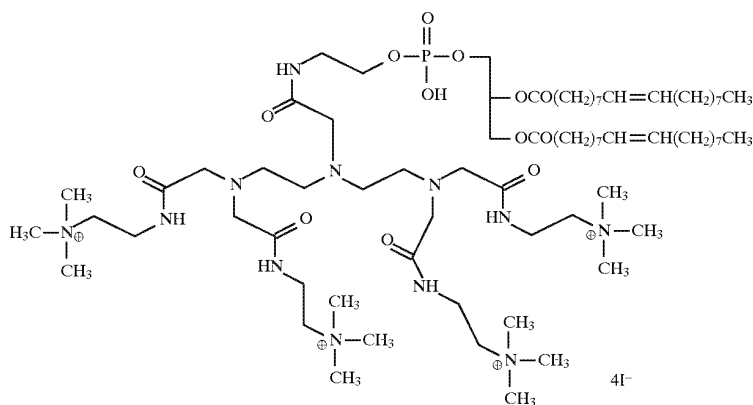
EXAMPLE 5C

N,N'-Bis(hexadecylaminocarbonylmethylene-N,N'-bis(trimethylammoniumethylaminocarbonyl methylene)ethylenediamine diiodide
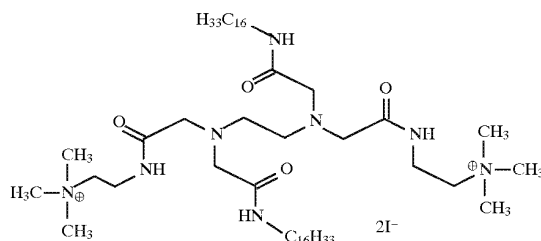
EXAMPLE 5D

N,N'-bis(hexadecyloxycarbonylmethylene)-N-(β-N, N, N-trimethylammoniumethylaminocarbonylmethylene)-N-methyl-N'-(carboxymethylene)ethylenediamine diiodide
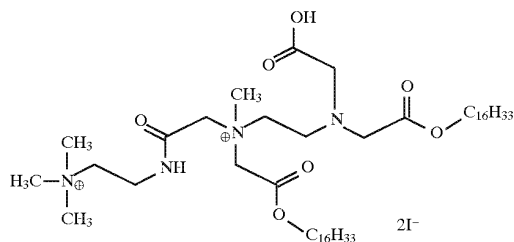
EXAMPLE 5E

N,N'-bis(hexadecylaminocarbonylmethylene)-N,N'-bis(β-N,N,N-trimethylammoniumethylaminocarbonyl methylene)-N,N'-dimethylethylenediamine tetraiodide
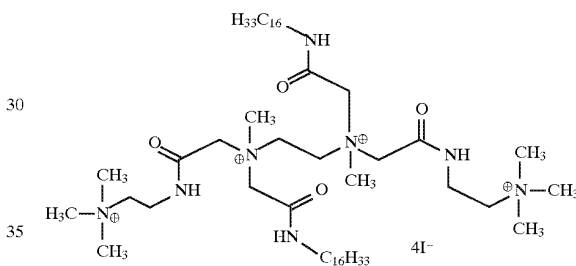
Formulations of this invention are subjected to various biological tests, the results of which correlate to useful therapeutic activity. These tests are useful in determining the ability of the present formulations to deliver intracellularly bioactive agents, including genetic material. These tests are useful also in determining the ability of the present formulations to treat genetic diseases, including diseases which involve a pathology or condition which is associated with loss or deterioration of immune competence.

EXAMPLE 6

The following examples are directed to the intracellular delivery of genetic material with cationic lipid compounds of the present invention and compounds disclosed in the prior art. The genetic material involved in these transfection studies is DNA that codes for Chloramphenicol Acetyl Transferase ("CAT"). The amount of expressed CAT (nanograms per mL (ng/mL)) was assayed using the Boehringer Mannheim CAT ELISAT™ kit, the results of which are tabulated in FIGS. 1 and 2.

LIPOFECTAMINE™ and LIPOFECTIN® were purchased from Gibco BRL, a division of Life Technologies, Inc. (Gaithersburg, Md.). LIPOFECTAMINE™ is a 3:1 liposome formulation of N-[2-({2,5-bis(3-aminopropyl) amino]-1-oxypentyl}amino)ethyl-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-1-propanaminium trifluoroacetate and dioleoylphosphatidylethanolamine ("DOPE"). LIPOFECTIN® is a liposome formulation of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride ("DOTMA") and

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DOPE. (See *Proc. Natl. Acad. Sci. USA*, Vol. 84, p. 7413 (1987).) TRANSFECTAM™ was purchased from Promega Corp (Madison, Wis.). TRANSFECTAM™ is a cationic lipopolyamine compound which comprises a spermine headgroup. (See *Proc. Natl. Acad. Sci.*, Vol. 86, p. 6982 (1989); *J. Neurochem.*, Vol. 54, p. 1812 (1990); and *DNA and Cell Biology*, Vol. 12, p. 553 (1993).) "DOTAP" refers to 1,2-dioleoyl-3-propyl-N,N,N-trimethylammonium halide.

EXAMPLE 6A

This example describes the biological testing of the compounds prepared in Examples 5C and 5D, Lipofectin, Lipofectamine and DOTAP and Transfectam in the absence of serum.

HeLa cells (American Type Culture Collection, Rockville, Md.) were cultured in EMEM media (Mediatech, Washington, D.C.). The cells were grown in 6-well plates (Becton Dickinson, Lincoln Park, N.J.) and at a density of 4×10^5 cells/well until they were 60–80% confluent in a VWR model 2500 CO_2 incubator (VWR, Philadelphia, Pa.). DNA (1.7 μg) was diluted to 50 μL in HEPES buffered saline (HBS) (HEPES 20 mM, 150 mM NaCl, pH 7.4) for each well to be infected. 10 μL solutions of the cationic lipid compounds prepared in Examples 5C and 5D, Lipofectin, Lipofectamine, DOTAP and Transfectam were each diluted to 50 μL in HBS. The DNA and lipid solutions were mixed by inverting and incubated at room temperature for 15 minutes. After incubating, each of the mixtures of DNA and cationic lipid (100 μL) was added to 1.9 mL of media without serum and mixed by inverting. The media was removed from the 6-well plates and replaced with the media containing lipid and DNA. The cells were then incubated in a CO_2 atmosphere at 37° C. for 5–6 hours. After incubating, the lipid/DNA media was removed and replaced with complete media. The cells were then incubated for 48–72 hours and the level of expressed protein was assayed. The results of the assay were measured using an SLT Labinstruments SPECTRA Shell plate reader (SLT, Salzburg, Austria) which was linked to a Centris 650 computer (Apple Computer, Inc., Cupertino, Calif.) and controlled using DeltaSoft II version 4.13s (Biometallics, Inc., Princeton, N.J.). The results of the assay are depicted in FIG. **1** which show increased expression of CAT when the cationic lipid compounds of the present invention are used to transfect cells, relative to compounds of the prior art. Accordingly, the experiments performed in this example demonstrate that the cationic lipid compounds of the present invention provide useful and improved transfection of cells with bioactive agents as compared to compounds of the prior art.

EXAMPLE 6B

This example describes the biological testing of the compound prepared in Example 5D, Lipofectin and Lipofectamine in the presence of serum.

HeLa cells were cultured in 4 mL of culture media as described above, except that the media was supplemented with enriched calf serum (Gibco BRL Life Technologies, Gaithersburg, Md.) and Penicillin/Streptomycin (Boehringer Mannheim Biochemicals (BMB), Indianapolis, Ind.). The cells were grown in 6-well plates (Becton Dickinson, Lincoln Park, N.J.) and at a density of 4×10^5 cells/well until they were 60–80% confluent in a VWR model 2500 CO_2 incubator (VWR, Philadelphia, Pa.). DNA (3.3 μg) was diluted to 100 μL in HEPES buffered saline (HBS) (HEPES 20 mM, 150 mM NaCl, pH 7.4) for each well to be infected.

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20 μL solutions were prepared of (1) the cationic lipid compound of Example 5D in combination with varying amounts of DOPE; (2) Lipofectin; and (3) Lipofectamine. These were diluted to 100 μL in HBS for each well. The compound of Example 5D and DOPE were combined in weight ratios of 5:1, 6:1, 7:1, 9:1, 11:1 and 14:1. The DNA and lipid solutions were mixed by inverting and incubated at room temperature for 15 minutes. After incubating, the mixtures of DNA and cationic lipid (200 μL) were added to each well (except for a HeLa (CELLS) standard to which no DNA with cationic lipid was added) and the mixtures were agitated by pipetting several times upwards and downwards. The cells were then incubated in a CO_2 atmosphere at 37° C. for 48–72 hours. After incubating, the protein level was assayed as described above.

The results of the assay are depicted in FIG. **2** which show increased expression of CAT when the cationic lipid compounds of the present invention are used to transfect cells, relative to compounds of the prior art. Particularly desirable transfection is observed in compositions which comprise a 6:1 ratio of the compound of Example 5D to DOPE. The experiments performed in this example demonstrate that the cationic lipid compounds of the present invention provide improved transfection of cells with bioactive agents as compared to compounds of the prior art.

EXAMPLE 7

In vivo experiments in rats were performed which demonstrate the high effectiveness of the present cationic lipid compounds to deliver intracellularly genetic material. The experiments demonstrate also the effectiveness of using ultrasound energy for targeting specific tissue in vivo with vesicular compositions containing genetic material.

Plasmid pSV β-gal (Promega, Madison, Wis.) which contains the β-galactosidase gene was combined with the cationic lipid compound prepared in Example 5D by mixing. The resulting mixture was injected into each of three Sprague Dawley rats (rats (A), (B) and (C)) via the tail vein. Rat (A) was not subjected to ultrasound. Ultrasonic energy was applied to the inside of the hind leg during injection for each of rats (B) and (C). After 48 hours, the rats were euthanized and the tissues were removed. The tissues were fixed for 72 hours in 2% formalin, sliced thin and placed in an X-gal solution. After 16 hours at 37° C., the tissues were inspected. The tissue from rat (A) exhibited a blue color which is indicative of general transfection. The tissue from rats (B) and (C) exhibited blue color only at the site where ultrasound energy was applied. This indicates that localization of gene expression can be achieved with the compounds and methods of the present invention.

EXAMPLE 8

A cationic lipid composition according to the present invention was prepared from six parts of the compound prepared in Example 5D and 1 part dipalmitoylphosphatidylethanolamine (DPPE) labeled with rhodamine (Avanti Polarlipids, Alabaster, Ala.). The cationic lipid composition was dissolved in ethanol and a Mansfield angioplasty catheter tip (Boston Scientific Corp., Watertown, Mass.) was dipped into the ethanolic formulation, removed and allowed to dry. This procedure was repeated three times. The coated catheter tips were then placed onto a Nikon light microscope equipped with a filter for rhodamine fluorescence. A control catheter, which was not coated with the cationic lipid composition, was also placed onto the light microscope. Fluorescence of the coated catheter tips was observed,

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whereas the control catheter tips did not fluoresce. This confirmed the presence of a coating of the lipid composition on each of the coated catheters. The coated catheter tips were then dipped into normal saline, water and human serum for varying periods of time and viewed under the light microscope. Fluorescence of the catheter tips was observed again. This demonstrated that the coating of the rhodamine-labeled lipid composition adhered to the surface of the catheters. Accordingly, lipid compositions of the present invention can be delivered to specific locations within the body by coating the compositions onto catheters which are then administered to a patient, as appropriate.

EXAMPLE 9

A cationic lipid formulation according to the present invention was prepared from DNA (5 μg) fluorescently labelled with fluorescein-12 DUTP (deoxyuracil triphosphate, commercially available from Boehringer Mannheim Biochemicals (BMB), Indianapolis, Ind.) using PCR, six parts of the compound prepared in Example 5D and 1 part DPPE. Catheters were subsequently dipped into ethanolic solutions of the cationic lipid formulation as described (for the compositions) in Example 8. A control catheter was not coated with the subject cationic lipid formulation. Fluorescence was induced and observed for the coated catheters as described in Example 8. No fluorescence was observed with the control catheter. This demonstrated that the cationic lipid formulations of the present invention adhere to the surface of catheters. Accordingly, lipid formulations of the present invention can be delivered to specific locations within the body by coating the formulations onto catheters which are then administered to a patient, as appropriate.

EXAMPLE 10

A cationic lipid formulation according to the present invention will be prepared from 6 parts of the compound of Example 5D, 1 part DPPE (dipalmitoyl-phosphatidylethanolamine) and 10 μg of plasmid DNA containing the gene for endothelial cell growth factor and a Respiratory Syncytial Virus RSV) growth factor. The formulation will be lyophilized, and 1 to 10 μg of the lyophilized formulation will be coated on a balloon of an angioplasty catheter. Coating will be accomplished by simply dipping the balloon into the formulation. The angioplasty catheter will be introduced into the left anterior descending coronary artery of a patient to cross the region of a hemodynamically significant stenosis. The catheter will be inflated to 6 atmospheres of pressure with the coated balloon. The stenosis will be alleviated and the lyophilized coating on the balloon will be deposited on the arterial wall. Transfection of endothelial cells results in localized production of endothelial cell growth factor. Healing of the arterial wall will be improved and fibroblast proliferation will be reduced, resulting also in lessened restenosis.

EXAMPLE 11

The procedure described in Example 10 will be repeated except that the surface of the catheter balloon will be modified to improve the binding of the lyophilized cationic lipid formulation according to the procedure described in K. Ishihara et al., *Journal of Biomedical Materials Research*, Vol. 27, pp. 1309–1314 (1993).

EXAMPLE 12

The procedure described in Example 10 will be repeated except that a vascular stent comprising, for example,

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DACRON™ (a polyester synthetic fiber) and/or wire mesh, is substituted for the angioplasty catheter. Improved endothelialization is obtained along the surface of the stent.

We claim:

1. A cationic lipid compound of the formula:

$$\underset{|}{(R_4\!-\!Y_3)_z} \tag{I}$$
$$Y_1\!-\!(R_1\!-\!X_1)_x\!-\!R_2\!-\![Y_2\!-\!R_3]_y\!-\!(X_1\!-\!R_1)_x\!-\!Y_1$$

wherein:

each of x, y and z is independently an integer from 0 to about 100;

each X_1 is independently —O—, —S—, —NR$_5$—, —C(=X$_2$)—, —C(=X$_2$)—N(R$_5$)—, —N(R$_5$)—C(=X$_2$)—, —C(=X$_2$)—O—, —O—C(=X$_2$)— or —X$_2$—(R$_5$X$_2$)P(=X$_2$)—X$_2$—;

each X_2 is independently O or S;

each Y_1 is independently a phosphate residue, N(R$_6$)$_a$—, S(R$_6$)$_a$—, P(R$_6$)$_a$— or —CO$_2$R$_6$, wherein a is an integer from 1 to 3;

each Y_2 is independently —N(R$_6$)$_b$—, —S(R$_6$)$_b$— or —P(R$_6$)$_b$—, wherein b is an integer from 0 to 2;

each Y_3 is independently a phosphate residue, N(R$_6$)$_a$—, S(R$_6$)$_a$—, P(R$_6$)$_a$— or —CO$_2$R$_6$, wherein a is an integer from 1 to 3;

each of R$_1$, R$_2$, R$_3$ and R$_4$ is independently alkylene of 2 to about 20 carbons;

each R$_5$ is independently hydrogen or alkyl of 1 to about 10 carbons; and

each R$_6$ is independently —[R$_7$—X$_3$]$_c$—R$_8$ or —R$_9$—[X$_4$—R$_{10}$]$_d$—Q, wherein:

each of c and d is independently an integer from 0 to about 100;

each Q is independently a phosphate residue, —N(R$_{11}$)$_q$, —S(R$_{11}$)$_q$, —P(R$_{11}$)$_q$ or —CO$_2$R$_{11}$, wherein q is an integer from 1 to 3;

each of X$_3$ and X$_4$ is independently —O—, —S—, —NR$_5$—, —C(=X$_2$)—, —C(=X$_2$)—N(R$_5$)—, —N(R$_5$)—C(=X$_2$)—, —C(=X$_2$)—O—, —O—C(=X$_2$)— or —X$_2$—(R$_5$X$_2$)P(=X$_2$)—X$_2$—;

each R$_7$ is independently alkylene of 2 to about 20 carbons;

each R$_8$ is independently hydrogen or alkyl of 1 to about 60 carbons;

each of R$_9$ and R$_{10}$ is independently alkylene of 2 to about 20 carbons; and

each R$_{11}$ is independently —[R$_7$—X$_3$]$_c$—R$_8$ or —R$_9$—[X$_4$—R$_{10}$]$_d$—W, wherein:

each W is independently a phosphate residue, —N(R$_{12}$)$_w$, —S(R$_{12}$)$_w$, —P(R$_{12}$)$_w$ or —CO$_2$R$_{12}$, wherein w is an integer from 1 to 3; and

R$_{12}$ is —[R$_7$—X$_3$]$_c$—R$_8$; with the proviso that the compound of formula (I) comprises at least two quaternary salts.

2. The compound according to claim **1** wherein said quaternary salt comprises a pharmaceutically-acceptable counter ion.

3. The compound according to claim **2** wherein said counter ion is selected from the group consisting of halide, R$_{13}$SO$_3^-$, R$_{13}$CO$_2^-$, phosphate, sulfite, nitrate, gluconate, guluronate, galacturonate, estolate and mesylate, wherein R$_{13}$ is hydrogen, alkyl of 1 to about 20 carbons or aryl of about 6 to about 10 carbons.

4. The compound according to claim **1** wherein said compound is in lyophilized form.

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5. The compound according to claim **1** wherein:

each of c, d, x, y and z is independently an integer from 0 to about 50;

each Q is independently a phosphate residue, $—CO_2R_{11}$ or $—N(R_{11})_q$, wherein q is 2 or 3; and

each W is independently a phosphate residue, $—CO_2R_{12}$ or $—N(R_{12})_w$, wherein w is 2 or 3.

6. The compound according to claim **5** wherein:

each of q and w is 3.

7. The compound according to claim **6** wherein:

each of c, d, x, y and z is independently an integer from 0 to about 20; and

X_2 is O.

8. The compound according to claim **7** wherein:

each of c, d, x, y and z is independently an integer from 0 to about 10; and

each of X_1, X_3 and X_4 is independently $—C(=O)—NR_5—$, $—NR_5—C(=O)—$, $—C(=O)—O—$ or $—O—C(=O)—$.

9. The compound according to claim **8** wherein:

each of c, d, x, y and z is an integer from 0 to about 5.

10. The compound according to claim **9** wherein:

each of R_1, R_2, R_3 and R_4 is independently straight chain alkylene of 2 to about 10 carbons or cycloalkylene of about 4 to about 10 carbons;

each R_5 is independently hydrogen or alkyl of 1 to about 4 carbons;

each R_7 is independently alkylene of 2 to about 10 carbons;

each R_8 is independently hydrogen or alkyl of 1 to about 40 carbons; and

each of R_9 and R_{10} is independently alkylene of 2 to about 10 carbons.

11. The compound according to claim **10** wherein:

each of R_1, R_2, R_3 and R_4 is independently straight chain alkylene of 2 to about 4 carbons or cycloalkylene of about 5 to about 7 carbons;

R_5 is hydrogen;

each R_7 is independently alkylene of 2 to about 4 carbons;

each R_8 is independently hydrogen or alkyl of 1 to about 20 carbons; and

each of R_9 and R_{10} is independently alkylene of 2 to about 4 carbons.

12. The compound according to claim **11** wherein:

each Y_1 is independently a phosphate residue, $N(R_6)_a—$ or $—CO_2R_6$;

Y_2 is $—N—(R_6)_b—$; and

each Y_3 is independently a phosphate residue, $N(R_6)_a—$ or $—CO_2R_6$.

13. The compound according to claim **12** wherein:

x is 1.

14. The compound according to claim **13** wherein:

y is 2 and z is 0.

15. The compound according to claim **14** wherein:

each Y_1 is independently $N(R_6)_a—$ or $—CO_2R_6$; and

R_6 is $—[R_7—X_3]_c—R_8$.

16. The compound according to claim **15** wherein:

each of R_1, R_2, R_3, R_4 and R_7 is independently methylene, ethylene or cyclohexylene; and

each R_8 is independently hydrogen or alkyl of about 1 to about 16 carbons.

17. The compound according to claim **16** wherein:

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a is 3; and

each c is independently 0 or 1.

18. The compound according to claim **17** wherein:

b is 1.

19. The compound according to claim **18** which is N,N'-bis(dodecyloxycarbonylmethylene)-N,N'-bis(β-N,N,N-trimethylammoniumethylaminocarbonylmethylene) ethylenediamine dihalide.

20. The compound according to claim **19** wherein said halide is chloride, bromide or iodide.

21. The compound according to claim **18** which is N,N'-bis(hexadecylaminocarbonylmethylene-N,N'-bis (trimethylammoniumethylaminocarbonylmethylene) ethylenediaminedihalide.

22. The compound according to claim **21** wherein said halide is chloride, bromide or iodide.

23. The compound according to claim **17** wherein:

b is 2.

24. The compound according to claim **23** which is N,N'-bis(dodecylaminocarbonylmethylene)-N,N'-bis(β-N,N,N-trimethylammoniumethylaminocarbonylmethylene)-N,N'-dimethylethylenediamine tetrahalide.

25. The compound according to claim **24** wherein said halide is chloride, bromide or iodide.

26. The compound according to claim **23** which is N,N'-bis(dodecylaminocarbonylmethylene)-N,N'-bis(β-N,N,N-trimethylammoniumethylaminocarbonylmethylene)-N,N'-dimethylcyclohexylene-1,4-diamine tetrahalide.

27. The compound according to claim **26** wherein said halide is chloride, bromide or iodide.

28. The compound according to claim **23** which is N,N'-bis(hexadecylaminocarbonylmethylene)-N,N'-bis(β-N,N,N-trimethylaxmmoniumethylaminocarbonylmethylene)-N,N'-dimethylethylenediamine tetrahalide.

29. The compound according to claim **28** wherein said halide is chloride, bromide or iodide.

30. The compound according to claim **17** wherein:

each b is independently 1 or 2.

31. The compound according to claim **30** which is N,N'-bis(hexadecyloxycarbonylmethylene)-N-(β-N,N,N-trimethylammoniumethylaminocarbonylmethylene)-N-methyl-N'-(carboxymethylene)ethylenediamine dihalide.

32. The compound according to claim **31** wherein said halide is chloride, bromide or iodide.

33. The compound according to claim **13** wherein:

y is 2 and z is 0 or 1.

34. The compound according to claim **33** wherein:

a is 3;

b is 2;

each c is independently 0 or 1; and

d is 1.

35. The compound according to claim **34** wherein:

R_{11} is $—[R_7—X_3]_c—R_8$.

36. The compound according to claim **35** wherein:

each of R_1, R_2, R_3, R_4 and R_7 is independently methylene or ethylene; and

each R_8 is independently hydrogen or alkyl of 1 to about 16 carbons.

37. The compound according to claim **36** which is N,N''-bis(hexadecylaminocarbonylmethylene)-N,N',N''-tris(β-N,N, N-trimethylammoniumethylaminocarbonylmethylene)-N,N',N''-trimethyldiethylenetriamine hexahalide.

38. The compound according to claim **37** wherein said halide is chloride, bromide or iodide.

39. The compound according to claim **13** wherein:

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y is 3 and z is 0.

40. The compound according to claim **39** wherein:

a is 3;

b is 2;

each c is independently 0 or 1; and

d is 1.

41. The compound according to claim **40** wherein:

each of R_1, R_2, R_3, R_4 and R_7 is independently methylene or ethylene;

each R_8 is independently hydrogen or alkyl of 1 to about 16 carbons;

each of R_9 and R_{10} is independently methylene or ethylene; and

R_{11} is methyl.

42. The compound according to claim **41** which is 1,1,7, 7 - t e t r a (β - N , N , N , N - tetramethylammoniumethylaminocarbonylmethylene)-4-hexadecylaminocarbonylmethylene-N,N',N'-trimethyl-1,4, 7-triazaheptane heptahalide.

43. The compound according to claim **42** wherein said halide is chloride, bromide or iodide.

44. The compound according to claim **13** wherein:

y is 3 and z is 0.

45. The compound according to claim **44** wherein:

a is 3;

b is 1;

c is 0; and

d is 1.

46. The compound according to claim **45** wherein:

each of R_1, R_2 and R_3 is independently methylene or ethylene;

each R_8 is independently hydrogen or methyl;

each of R_9 and R_{10} is independently methylene or ethylene; and

R_{11} is methyl.

47. The compound according to claim **46** which is N,N, N '' , N '' - t e t r a (β - N , N , N - trimethylammoniumethylaminocarbonyl-methylene)-N'-(1, 2 - d i o l e o y l g l y c e r o - 3 - p h o s p h o e t h a n o l a m i n o c a r b o n y l m e t h y l e n e) diethylenetriamine tetrahalide.

48. The compound according to claim **47** wherein said halide is chloride, bromide or iodide.

49. A cationic lipid composition comprising the cationic lipid compound according to claim **1**.

50. The cationic lipid composition according to claim **49** wherein said composition is lyophilized.

51. The cationic lipid composition according to claim **49** which is selected from the group consisting of micelles, liposomes and mixtures thereof.

52. The cationic lipid composition according to claim **51** further comprising an amphipathic compound for stabilizing the composition.

53. The cationic lipid composition according to claim **52** further comprising a gas, a precursor to a gas or a mixture thereof.

54. The cationic lipid composition according to claim **53** wherein said gas or said precursor to a gas is selected from the group consisting of perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluorocyclobutane, perfluoropentane, perfluorohexane, and mixtures thereof.

55. The cationic lipid composition according to claim **53** which comprises a mixture of a gas and a precursor to a gas.

56. The cationic lipid composition according to claim **54** wherein said gas or gaseous precursor further comprises nitrogen.

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57. The cationic lipid composition according to claim **49** further comprising a gas, a precursor to a gas, or a mixture thereof, and an amphipathic compound for stabilizing the composition.

58. The cationic lipid composition according to claim **57** wherein said amphipathic compound comprises a lipid.

59. The cationic lipid composition according to claim **58** which is selected from the group consisting of suspensions, emulsions, and vesicle compositions.

60. The cationic lipid composition according to claim **59** which comprises a cationic vesicle composition.

61. The cationic vesicle composition according to claim **60** wherein said vesicles are selected from the group consisting of unilamellar vesicles, oligolamellar vesicles and multilamellar vesicles.

62. The cationic vesicle composition according to claim **61** wherein said lipid is a polyrnerizable lipid.

63. The cationic vesicle composition according to claim **61** further comprising polyethyleneglycol.

64. The cationic vesicle composition according to claim **61** wherein said vesicles comprise unilamellar vesicles.

65. The cationic vesicle composition according to claim **64** wherein said vesicles comprise a monolayer.

66. The cationic vesicle composition according to claim **65** wherein said lipid is a phospholipid and said gas or said precursor to a gas is sulfur hexafluoride.

67. The cationic vesicle composition according to claim **65** wherein said lipid is a phospholipid and said gas or gaseous precursor is perfluoropentane.

68. The cationic vesicle composition according to claim **65** wherein said lipid is a phospholipid and said gas or gaseous precursor is perfluoropropane.

69. The cationic vesicle composition according to claim **64** wherein said vesicles comprise a bilayer.

70. The cationic vesicle composition according to claim **69** wherein said lipid is a phospholipid and said gas or gaseous precursor is sulfur hexafluoride.

71. The cationic vesicle composition according to claim **69** wherein said lipid is a phospholipid and said gas or gaseous precursor is perfluoropentane.

72. The cationic vesicle composition according to claim **69** wherein said lipid is a phospholipid and said gas or gaseous precursor is perfluoropropane.

73. The cationic vesicle composition according to claim **59** wherein said vesicles are selected from the group consisting of oligolamellar and multilamellar vesicles.

74. The cationic vesicle composition according to claim **73** wherein said vesicles comprise monolayers.

75. The cationic vesicle composition according to claim **74** wherein said lipid is a phospholipid and said gas or gaseous precursor is sulfur hexafluoride.

76. The cationic vesicle composition according to claim **74** wherein said lipid is a phospholipid and said gas or said precursor to a gas is perfluoropentane.

77. The cationic vesicle composition according to claim **74** wherein said lipid is a phospholipid and said gas or gaseous precursor is perfluoropropane.

78. The cationic vesicle composition according to claim **73** wherein said vesicles comprise bilayers.

79. The cationic vesicle composition according to claim **78** wherein said lipid is a phospholipid and said gas or gaseous precursor is sulfur hexafluoride.

80. The cationic vesicle composition according to claim **78** wherein said lipid is a phospholipid and said gas or gaseous precursor is perfluoropentane.

81. The cationic vesicle composition according to claim **78** wherein said lipid is a phospholipid and said gas or gaseous precursor is perfluoropropane.

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82. The cationic lipid composition according to claim **58** wherein said lipids comprise unilamellar lipids, oligolamellar lipids or multilamellar lipids.

83. The cationic lipid composition according to claim **82** wherein said lipids are polymerizable lipids.

84. The cationic lipid composition according to claim **82** further comprising polyethyleneglycol.

85. The cationic lipid composition according to claim **82** wherein said lipids comprise unilamellar lipids.

86. The cationic lipid composition according to claim **85** wherein said lipids are in the form of a monolayer.

87. The cationic lipid composition according to claim **86** wherein said lipids are phospholipids and said gas or gaseous precursor is sulfur hexafluoride.

88. The cationic lipid composition according to claim **86** wherein said lipids are phospholipids and said gas or gaseous precursor is perfluoropentane.

89. The cationic lipid composition according to claim **86** wherein said lipids are phospholipids and said gas or gaseous precursor is perfluoropropane.

90. The cationic lipid composition according to claim **85** wherein said lipids are in the form of a bilayer.

91. The cationic lipid composition according to claim **90** wherein said lipids are phospholipids and said gas or gaseous precursor is sulfur hexafluoride.

92. The cationic lipid composition according to claim **90** wherein said lipids are phospholipids and said gas or gaseous precursor is perfluoropentane.

93. The cationic lipid composition according to claim **90** wherein said lipids are phospholipids and said gas or gaseous precursor is perfluoropropane.

94. The cationic lipid composition according to claim **82** wherein said lipids comprise oligolamellar lipids or multilamellar lipids.

95. The cationic lipid composition according to claim **94** wherein said lipids are in the form of monolayers.

96. The cationic lipid composition according to claim **95** wherein said lipids are phospholipids and said gas or gaseous precursor is sulfur hexafluoride.

97. The cationic lipid composition according to claim **95** wherein said lipids are phospholipids and said gas or gaseous precursor is perfluoropentane.

98. The cationic lipid composition according to claim **95** wherein said lipids are phospholipids and said gas or gaseous precursor is perfluoropropane.

99. The cationic lipid composition according to claim **94** wherein said lipids are in the form of bilayers.

100. The cationic lipid composition according to claim **99** wherein said lipids are phospholipids and said gas or gaseous precursor is sulfur hexafluoride.

101. The cationic lipid composition according to claim **99** wherein said lipids are phospholipids and said gas or gaseous precursor is perfluoropentane.

102. The cationic lipid composition according to claim **99** wherein said lipids are phospholipids and said gas or gaseous precursor is perfluoropropane.

103. A cationic lipid formulation for the intracellular delivery of a bioactive agent comprising, in combination with a bioactive agent, a cationic lipid composition which comprises the cationic lipid compound according to claim **1**.

104. The lipis formulation according to claim **103** wherein said bioactive agent comprises genetic material.

105. The lipid formulation according to claim **103** which is selected from the group consisting of micelles, liposomes and mixtures thereof.

106. The lipid formulation according to claim **105** comprising said bioactive agent entrapped within said micelles or liposomes.

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107. The lipid formulation according to claim **103** further comprising a gas, a gaseous precursor or a mixture thereof.

108. The lipid formulation according to claim **107** wherein said gas, gaseous precursor or mixture thereof is selected from the group consisting of perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluorocyclobutane, perfluoropentane, perfluorohexane and mixtures thereof.

109. The lipid formulation according to claim **108** wherein said gas, gaseous precursor or mixture thereof further comprises nitrogen.

110. The process for the preparation of a cationic lipid formulation for the intracellular delivery of a bioactive agent comprising combining together a bioactive agent and a cationic lipid composition which comprises the cationic lipid compound according to claim **1**.

111. The process according to claim **110** wherein said bioactive agent comprises genetic material.

112. The process according to claim **110** wherein said formulation is lyophilized.

113. The process according to claim **110** wherein said composition is selected from the group consisting of micelles, liposomes and mixtures thereof.

114. The process according to claim **113** comprising entrapping said bioactive agent within said micelles or liposomes.

115. The process according to claim **110** wherein said composition further comprises a gas, a gaseous precursor or a mixture thereof.

116. The process according to claim **115** wherein said gas, gaseous precursor or mixture thereof is selected from the group consisting of perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluorocyclobutane, perfluoropentane, perfluorohexane and mixtures thereof.

117. The process according to claim **116** wherein said gas, gaseous precursor or mixture thereof further comprises nitrogen.

118. A method for delivering intracellularly a bioactive agent comprising contacting a cell with a cationic lipid composition which comprises the cationic lipid compound according to claim **1** and the bioactive agent.

119. The method according to claim **118** wherein said composition is reconstituted from a lyophilized composition.

120. The method of claim **118** wherein said composition is selected from the group consisting of micelles, liposomes and mixtures thereof.

121. The method of claim **120** comprising said bioactive agent entrapped within said micelles or liposomes.

122. The method of claim **118** wherein said bioactive agent comprises genetic material.

123. The method of claim **122** wherein said genetic material is selected from the group consisting of polynucleotide, DNA, RNA, polypeptide and mixtures thereof.

124. The method according to claim **118** wherein said composition further comprises a gas, a gaseous precursor or a mixture thereof.

125. The method according to claim **124** wherein said gas, gaseous precursor or mixture thereof is selected from the group consisting of perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluorocyclobutane, perfluoropentane, perfluorohexane and mixtures thereof.

126. The method according to claim **125** wherein said gas, gaseous precursor or mixture thereof further comprises nitrogen.

127. The cationic vesicle comprising a cationic lipid compound according to claim **1** and a gas or gaseous precursor.

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128. The cationic vesicle according to claim **127** which is selected from the group consisting of unilamellar vesicles, oligolamellar vesicles and multilamellar vesicles.

129. The cationic vesicle according to claim **128** which comprises unilamellar vesicles.

130. The cationic vesicle according to claim **129** which comprises a monolayer.

131. The cationic vesicle according to claim **129** which comprises a bilayer.

132. The cationic vesicle according to claim **128** which is selected from the group consisting of oligolamellar vesicles and multilamellar vesicles.

133. The cationic vesicle according to claim **132** which comprises monolayers.

134. The cationic vesicle according to claim **132** which comprises bilayers.

135. The cationic vesicle according to claim **132** further comprising an amphipathic compound for stabilizing the vesicle.

136. The cationic vesicle according to claim **135** wherein said amphipathic compound comprises a lipid.

137. The cationic vesicle according to claim **136** wherein said lipid is a phospholipid and said gas or gaseous precursor is sulfur hexafluoride.

138. The cationic vesicle according to claim **136** wherein said lipid is a phospholipid and said gas or gaseous precursor is perfluoropentane.

139. The cationic vesicle according to claim **136** wherein said lipid is a phospholipid and said gas or gaseous precursor is perfluoropropane.

140. The cationic vesicle according to claim **136** wherein said lipid is a polymerizable lipid.

141. The cationic vesicle according to claim **136** further comprising polyethyleneglycol.

142. A cationic lipid compound of the formula
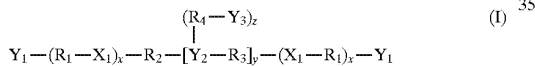
$$Y_1-(R_1-X_1)_x-R_2-[Y_2-R_3]_y-(X_1-R_1)_x-Y_1 \quad\overset{(R_4-Y_3)_z}{\underset{|}{}} \qquad (I)$$

wherein:

each of x, y and z is independently an integer from 0 to about 100;

each X_1 is independently —O—, —S—, —NR$_5$—, —C(=X$_2$)—, —C(=X$_2$)—N(R$_5$)—, —N(R$_5$)—C(=X$_2$)—, —C(=X$_2$)—O—, —O—C(=X$_2$)— or —X$_2$—(R$_5$X$_2$)P(=X$_2$)—X$_2$—;

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each X_2 is independently O or S;

each Y_1 is independently a phosphate residue, N(R$_6$)$_a$—, S(R$_6$)$_a$—, P(R$_6$)$_a$— or —CO$_2$R$_6$, wherein a is an integer from 1 to 3;

each Y_2 is independently —N(R$_6$)$_b$—, —S(R$_6$)$_b$— or —P(R$_6$)$_b$—, wherein b is an integer from 0 to 2;

each Y_3 is independently a phosphate residue, N(R$_6$)$_a$—, S(R$_6$)$_a$—, P(R$_6$)$_a$— or —CO$_2$R$_6$, wherein a is an integer from 1 to 3;

each of R_1, R_2, R_3 and R_4 is independently alkylene of 1 to about 20 carbons;

each R_5 is independently hydrogen or alkyl of 1 to about 10 carbons; and

each R_6 is independently —[R$_7$—X$_3$]$_c$—R$_8$ or —R$_9$—[X$_4$—R$_{10}$]$_d$—Q, wherein:

each of c and d is independently an integer from 0 to about 100;

each Q is independently a phosphate residue, —N(R$_{11}$)$_q$, —S(R$_{11}$)$_q$, —P(R$_{11}$)$_q$ or —CO$_2$R$_6$, wherein q is an integer from 1 to 3;

each of X_3 and X_4 is independently —O—, —S—, —NR$_5$—, —C(=X$_2$)—, —C(=X$_2$)—N(R$_5$)—, —N(R$_5$)—C(=X$_2$)—, —C(=X$_2$)—O—, —O—C(=X$_2$)— or —X$_2$—(R$_5$X$_2$)P(=X$_2$)—X$_2$—;

each R_7 is independently alkylene of 1 to about 20 carbons;

each R_8 is independently hydrogen or alkyl of 1 to about 40 carbons;

each of R_9 and R_{10} is independently alkylene of 1 to about 20 carbons; and

each R_{11} is independently —[R$_7$—X$_3$]$_c$—R$_8$ or —R$_9$—[X$_4$—R$_{10}$]$_d$—W, wherein:

each W is independently a phosphate residue, —N(R$_{12}$)$_w$, —S(R$_{12}$)$_w$, —P(R$_{12}$)$_w$, or —CO$_2$R$_6$, wherein w is an integer from 1 to 3; and

R_{12} is —[R$_7$—X$_3$]$_c$—R$_8$; with the proviso that the compound of formula (I) comprises at least one quaternary salt.

143. The compound according to claim **142** wherein said compound is in lyophilized form.

*   *   *   *   *

JA00936

MRNA-GEN-00222946

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,830,430

DATED   : Nov. 3, 1998

INVENTOR(S) : Unger et al.

Page 1 of 6

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

On the cover page, second column, under "OTHER PUBLICATIONS", at "Villanueva et al.", please delete "Patters" and insert --Patterns-- therefor.

On the cover page, second column, under "OTHER PUBLICATIONS", at "Keller et al.", second line thereof, please delete "Microcirulation" and insert --Microcirculation-- therefor.

On page 5, first column, under "OTHER PUBLICATIONS", at "Shiina et al.", please delete "Hyperthermiaby" and insert --Hyperthermia by-- therefor.

On page 5, first column, under "OTHER PUBLICATIONS", at "Poznansky et al.", please delete "Biologica" and insert --Biological-- therefor.

On page 5, second column, under "OTHER PUBLICATIONS", at "Ter-Pogossia", please delete "Ter-Pogossia" and insert --Ter-Pogossian,-- therefor.

On page 5, second column, under "OTHER PUBLICATIONS", at "San", please delete "Toxicitiy" and insert --Toxicity-- therefor.

On page 6, first column, under "OTHER PUBLICATIONS", at "Behr, J.", second line thereof, please delete "lipopolyamine-caoted" and insert --lipopolyamine-coated" therefor.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO.   : 5,830,430   Page 2 of 6
DATED   : Nov. 3, 1998
INVENTOR(S) : Unger et al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

On page 6, second column, under "OTHER PUBLICATIONS", at "Stel'mashok et al.", please delete "Stel'mashok" and insert --Stelmashok-- therefor.

In column 3, line 18, please delete "$_{or}-_{CO2}R_6$," and insert --or CO_2R_6,-- therefor.

In column 3, line 51, please delete "—$[R_7$—$X_3]_{c-R8}$" and insert -- —$[R_7$—$X_3]_c$—R_8-- therefor.

In column 5, line 5, please delete "R_{11}, is" and insert --R_{11} is-- therefor.

In column 6, line 49, please delete "rylthio" and insert --arylthio-- therefor.

In column 9, line 3, please delete "generally, concentric" and insert --generally concentric-- therefor.

In column 12, line 35, please delete "—C(=O)—NR_6—" and insert -- —C(=O)—NR_5— -- therefor.

In column 14, line 62, please delete "$[X_4$—$R_1]_d$—Q" and insert -- $[X_4$—$R_{10}]_d$—Q -- therefor.

In column 19, line 60, please delete "*Macro molecular*" and insert --*Macromolecular*-- therefor.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,830,430   Page 3 of 6

DATED   : Nov. 3, 1998

INVENTOR(S) : Unger et al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

In column 20, line 55, please delete "resent" and insert --present-- therefor.

In column 20, lines 57-58, please delete "fluoro surfactant" and insert --fluorosurfactant-- therefor.

In column 22, line 5, please delete "quipment" and insert --equipment-- therefor.

In column 22, line 9, please delete "ompositions" and insert --compositions-- therefor.

In column 22, line 9, please delete "resent" and insert --present-- therefor.

In column 27, line 5, please delete "intra ocular" and insert --intraocular-- therefor.

In column 27, line 5, please delete "trans epithelial" and insert --transepithelial-- therefor.

In column 29, line 29, please delete "(dodecylaminocarbonylmethylene)-N,N-'-bis" and insert --(dodecylaminocarbonylmethylene)-N,N'-bis-- therefor.

In column 30, line 35, please delete "0.59" and insert --0.5%-- therefor.

In column 30, line 61, please delete "(hexadecylaminocarbonylmethylene)-N,N'N''-tris(β-" and insert -- (hexadecylaminocarbonylmethylene)-N,N',N''-tris(β- -- therefor.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,830,430

DATED   : Nov. 3, 1998

INVENTOR(S) : Unger et al.

Page 4 of 6

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

In column 35, line 17, please delete "0°-50° C" and insert --0°-5° C-- therefor.

In column 38, line 57, please delete "CAT ELISAT™" and insert --CAT ELISA™-- therefor.

In column 42, line 57, claim 2, please delete "claim 1" and insert -- claim 142 -- therefor.

In column 42, line 66, claim 4, please delete "claim 1" and insert -- claim 142 -- therefor.

In column 43, line 1, claim 5, please delete "claim 1" and insert -- claim 142 -- therefor.

In column 43, line 48, claim 12, please delete "claim 11" and insert -- claim 142 -- therefor.

In column 44, line 14, claim 21, please delete "ethylenediaminedihalide" and insert --ethylenediamine dihalide-- therefor.

In column 44, line 34, claim 28, please delete "N-trimethylaxmmoniumethylamino-carbonylmethylene)-N" and insert --N-trimethylammoniummethylaminocarbonylmethylene)-N therefor.

In column 45, line 18, claim 42, please delete "hexadecylaminocarbonylmethylene-N,N',N'-trimethyl-1,4" and insert --hexadecylaminocarbonylmethylene-N,N',N''-trimethyl-1,4-- therefor.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,830,430   Page 5 of 6

DATED   : Nov. 3, 1998

INVENTOR(S) : Unger et al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

In column 45, line 46, claim 49, please delete "claim 1" and insert -- claim **142** -- therefor.

In column 46, line 16, claim 62, please delete "polymerizable" and insert --polymerizable-- therefor.

In column 47, line 59, claim 103, please delete "claim 1" and insert -- claim **142** -- therefor.

In column 47, line 60, claim 104, please delete "lipis" and insert --lipid-- therefor.

In column 48, line 12, claim 110, please delete "The process for" and insert --A process for-- therefor.

In column 48, line 15, claim 110, please delete "claim 1" and insert -- claim **142** -- therefor.

In column 48, line 39, claim 118, please delete "claim 1" and insert -- claim **142** -- therefor.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO.   : 5,830,430

DATED   : Nov. 3, 1998

INVENTOR(S) : Unger et al.

Page 6 of 6

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

In column 48, line 66, claim 127, please delete "claim 1" and insert -- claim **142** -- therefor.

Signed and Sealed this

Twenty-third Day of November, 1999

Attest:

Q. TODD DICKINSON

Attesting Officer

Acting Commissioner of Patents and Trademarks

JOINT APPENDIX 33

news feature
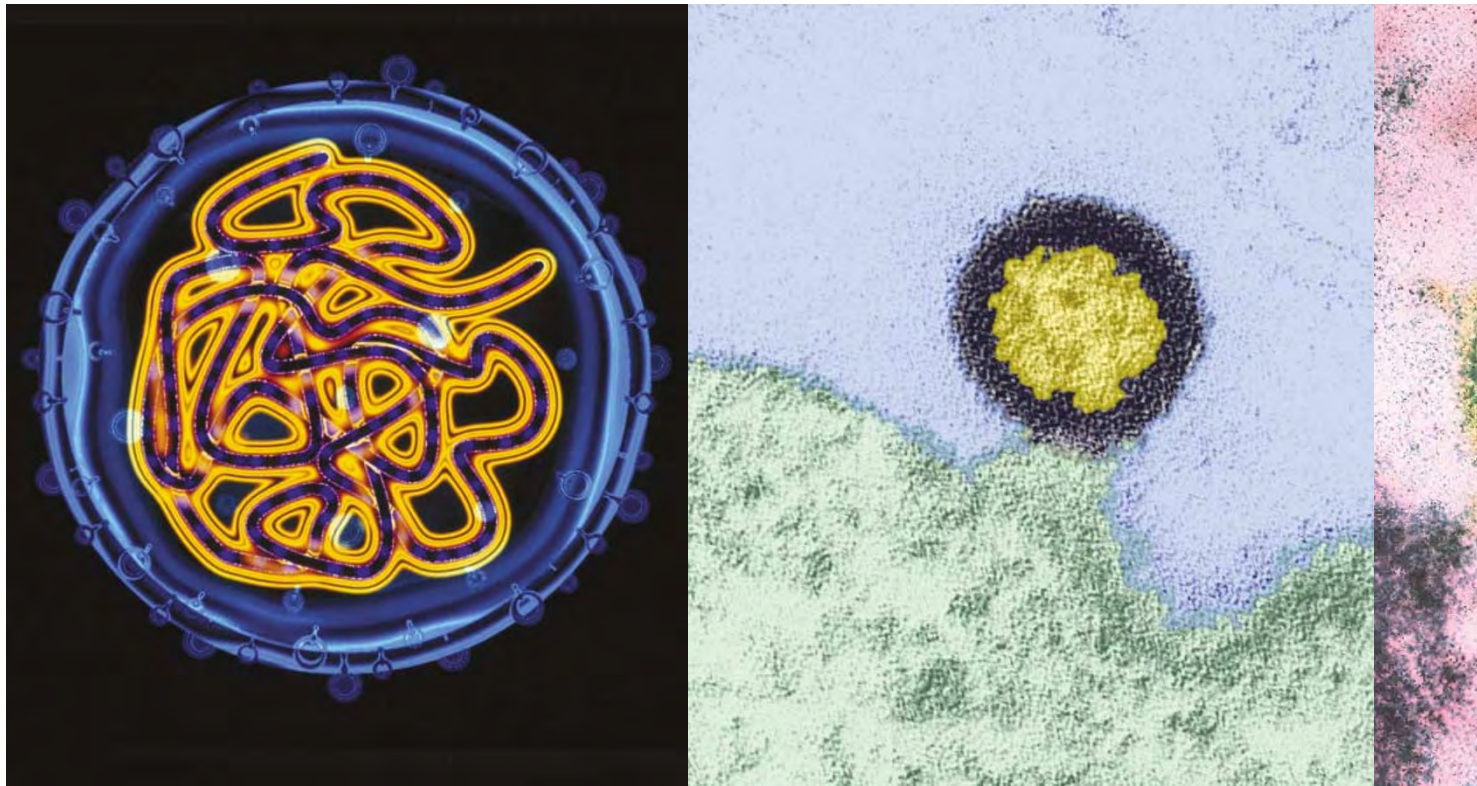
RNA to the rescue?

Disease therapies based on a technique for gene silencing called RNA interference are racing towards the clinic. Erika Check investigates molecular medicine's next big thing.

Medicine's molecular revolution is overdue. By now, enthusiasts led us to believe, gene therapy and related treatments should have transformed clinical practice. Diseases, they told us, would be cured at their genetic roots, by repairing defective human DNA or by disabling the genes of infectious microbes. But it has proved frustratingly difficult to make these methods work in the clinic — if you get sick, your doctor will probably still treat you with the pills and potions of old-fashioned medicinal chemistry.

Given this chastening experience, you would expect experts to be cautious about the prospects of molecular medicine's latest hope — a gene-silencing mechanism known as RNA interference, or RNAi. But instead, researchers can barely contain their enthusiasm. "Right now, everybody's excited," says Anastasia Khvorova, director of biology with Dharmacon in Lafayette, Colorado, a company that supplies RNAi technologies to researchers and companies that develop therapeutics.

The term RNAi was coined just five years ago, in a paper documenting the phenomenon in the nematode worm *Caenorhabditis*

elegans[1]. Yet doctors and biotech executives are now talking about beginning human trials within the next two or three years — an astonishing rate of progress. Part of the excitement stems from the knowledge that, unlike techniques such as gene therapy, RNAi is a natural defence mechanism that is thought to have evolved to protect organisms from viral diseases.

Dicey defence

Many viruses have a genetic blueprint made from RNA, rather than DNA. When they infect a cell, they make double-stranded copies of their genetic material. In response, the RNAi pathway strikes back. An enzyme known as Dicer first chops the double-stranded viral RNA into small segments of genetic code, each around 22 'letters' long. These segments, known as small interfering RNAs, or siRNAs, then separate into single strands and some bind to intact stretches of single-stranded viral RNA. Finally, proteins target this tagged viral RNA and destroy it[2]. As a result, RNAi shuts off key viral genes, potentially nipping infections in the bud.

Biologists are exploiting RNAi as an experimental tool to find out what genes do.

When a gene is activated, its sequence is read to produce messenger RNA (mRNA), which contains the information necessary to manufacture a particular protein. So by using siRNAs or double-stranded RNAs that correspond to a specific mRNA sequence, researchers can trick a cell into destroying this mRNA and silencing the gene in question.

As soon as it became obvious that the phenomenon operates in mammals[3] as well as in lower organisms, clinicians pricked up their ears. In theory, RNAi could be used to treat any disease — forms of cancer, for instance — that is linked to an overactive gene or genes. But for the time being, most of the clinical interest lies in applying RNAi in its natural role: as a means of combating pathogenic viruses by disabling their RNA.

One of the obvious targets is HIV — a virus for which there is no cure and no vaccine. Last year, for instance, molecular virologist Bryan Cullen of Duke University Medical Center in Durham, North Carolina, introduced siRNAs against two HIV genes into the human immune cells that are destroyed by the virus. The siRNAs allowed these cells to resist viral replication better than those that had not been triggered to undergo RNAi

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JA00943

news feature
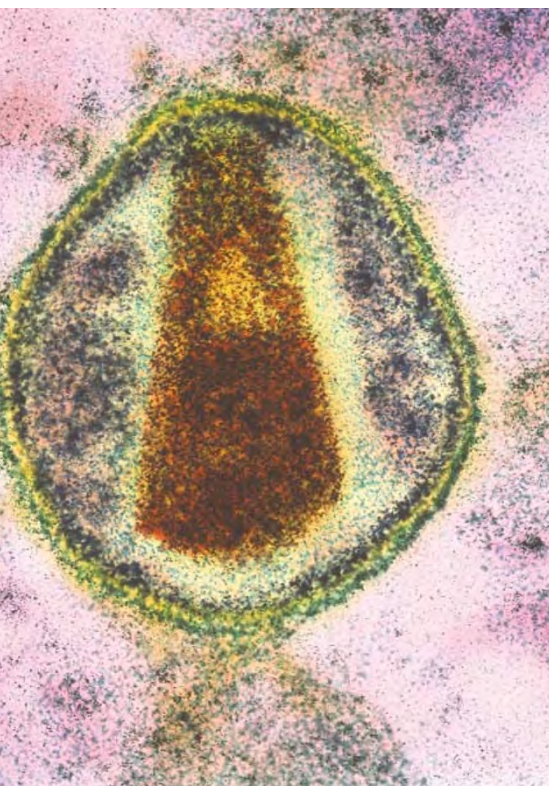
Combating the incurable: researchers are testing the idea that the RNAi pathway, which shuts down the genes of invading viruses, can block the replication of hepatitis C virus (far left, RNA shown in yellow) and HIV (left and middle left).

(ref. 4). Meanwhile, other researchers have shown that, in cultures of human cells, RNAi can similarly combat viruses as diverse as respiratory syncitial virus[5], and those that cause influenza[6] and polio[7].

RNAi may work like a charm in petri dishes — but what about in live animals? Mark Kay, a geneticist at Stanford University in California, addressed this question by fusing a genetic sequence from the hepatitis C virus to a gene for the enzyme luciferase, which stimulates a reaction that emits light. When Kay injected the fused gene into mouse livers, he could track its location by detecting the glow. And when the mice were treated with siRNAs targeted against the hepatitis C gene, this glow dimmed dramatically[8]. Hepatitis C doesn't make mice sick, but Kay and his colleagues have since gone on to show that RNAi can drastically reduce signs of infection by hepatitis B (ref. 9), which can damage the animals' livers.

Firm plans

Results such as these are attracting intense commercial interest. In August, Kay announced that he has licensed his work on hepatitis C to a company called Avocel in Sunnyvale, California, which aims to develop RNAi therapies against the disease. Other RNA pioneers are lining up with their own start-up biotech firms. For instance, Phillip Sharp of the Massachusetts Institute of Technology in Cambridge, who shared the Nobel Prize in Physiology or Medicine for his earlier work on RNA splicing, is one of the co-founders of Alnylam Pharmaceuticals, also based in Cambridge.

In June, this company merged with Ribopharma of Kulmbach in Germany.

Both Avocel and Alnylam are planning to begin clinical trials as early as 2005. And hot on their heels is Sirna Therapeutics of Boulder, Colorado, which has already raised US$43 million from investors. Sirna aims to develop therapies for hepatitis C and an eye condition called macular degeneration. For now, these companies are maintaining amicable relations. But the situation could get messier as RNAi moves towards the clinic, because patent offices around the world have not yet decided who owns the rights to some key RNAi-based technologies.

Before worrying about the ownership of key intellectual property, however, scientists must figure out how to make RNAi therapies work. They are facing some formidable technical barriers, chief among which is the problem of getting siRNAs into the right cells. This is not a trivial issue, because RNA is rapidly broken down in the bloodstream, and our cells don't readily absorb it through their membranes. And even when RNA gets into its target cell, scavenger proteins quickly chew it up. "The major hurdle right now is delivery, delivery, delivery," says Sharp.

Researchers are exploring a variety of ways to combat the problem. Some involve techniques developed to facilitate an older technology known as 'antisense'. The idea behind antisense is to muffle a cell's single-stranded mRNA — the 'sense' strand — using a piece of antisense RNA with a 'complementary' sequence that binds tightly to the mRNA. This, the theory goes, should prevent the mRNA from being translated into protein. Scientists tried a variety of ways to get the antisense RNAs into cells — for example, they packed the RNA inside fatty globules, called liposomes, which can cross cell membranes. But antisense has not performed well in clinical trials, partly because these delivery systems were not particularly effective. Khvorova believes that the medical benefits of RNAi will be huge if the delivery issues can be resolved. "But we've looked at a lot of the delivery methods that have been used for antisense, and so far I haven't been impressed," she says.

Harmless HIV

Another option is to use a harmless virus as a vector to ferry RNAi-triggering genes into their target cells. Molecular biologist John Rossi of the Beckman Research Institute of the City of Hope Medical Center in Duarte, California, is experimenting with one such vector, based on a version of HIV from which the disease-causing genes have been stripped. Together with colleagues led by Ramesh Akkina of Colorado State University in Fort Collins, Rossi engineered this vector to contain sequences encoding siRNAs targeted against HIV genes. The researchers used their vector to infect the human stem cells that develop into immune cells. Next, they either grew the cells into mature cells in the lab, or injected them into mice from a special strain that accepts human transplants. In both cases, the mature immune cells fought off HIV when researchers tried to infect them with disease-causing HIV in culture dishes[10].

Rossi hopes that a similar technique could work in human patients with HIV. Doctors

In mice containing a glowing version of a hepatitis C gene (left), a small interfering RNA (siRNA) against the gene reduces liver fluorescence (middle), but an unrelated copy of the siRNA (right) does not.

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news feature

would extract stem cells from a patient's bone marrow, infect them with the RNAi-triggering vector, and then put them back into the patient. Rossi is now working to perfect this technique in mice, and is also beginning tests in rhesus monkeys to ensure that the treatment has no unwanted side effects. He hopes to convince the US Food and Drug Administration to authorize a clinical trial in the next two or three years. "I think when we get all this data compiled we'll have a fairly free road into a stem-cell trial," Rossi predicts.

This may be so, but there are nagging safety concerns about vectors made from viruses in the same family as HIV, which are called retroviruses. This is due to the fact that retroviruses work by forcing their way into a cell's own DNA. If the vector lands in the wrong place it can damage important genes and even cause cancer. These concerns were borne out by last year's revelation that a retroviral vector had triggered leukaemia in some children in a gene-therapy trial[11]. Because of these concerns, Rossi says that he will not use stem cells in his first clinical trial. Instead, he will initially treat mature immune cells, because these cells are less likely to grow out of control.

Safe delivery

Kay, meanwhile, is pinning his hopes for an RNAi vector on a virus known as adeno-associated virus, or AAV. He has already used AAV-based vectors in clinical trials of gene therapy against haemophilia[12]. AAV does not cause disease in people, and so far there has been no cause for any serious safety concern — even though AAV can also integrate into a cell's own DNA.

Another important question mark hanging over RNAi is its specificity. Before regulators give the go-ahead for a clinical trial, scientists need to prove that that RNAi will not shut down vital human genes as well as the target viral sequences.

Some studies on specificity have yielded encouraging results. In May this year, for instance, researchers led by Patrick Brown of Stanford University reported on experiments in which they engineered human kidney cells to produce a fluorescent protein. They shut down the gene for this glowing protein by using RNAi, and then used DNA microarrays to monitor some 20,000 other genes — none of which seemed to be affected by the treatment[13].

But just a couple of weeks later, researchers with Rosetta Inpharmatics in Kirkland, Washington, cast a shadow over this rosy picture. The Rosetta team, led by Aimee Jackson and Steven Bartz, used a range of different siRNAs to target two genes in cultured human cells. Disturbingly, the treatment caused changes in the expression of dozens of other genes. Depending on the precise sequence of the siRNA concerned, a different range of 'off-target' genes seemed to be affected[14].

Little helpers: Mark Kay hopes to use harmless viruses to deliver RNAi therapy to patients.

Jackson and Bartz are not sure why their results were so different from those obtained by Brown's team, but one possible explanation is that they used larger doses of siRNA. The Rosetta researchers also tested for off-target effects sooner after beginning their experiment than other groups have in their studies. But whatever the explanation, the findings have shaken up the RNAi camp. "We've had some really lively discussions," says Bartz.

New data from a group at Case Western Reserve University in Cleveland, Ohio, seem to support the Rosetta findings[15]. Last week, Bryan Williams and his colleagues reported that when they introduced siRNAs into cells, certain genes that are part of the interferon

Going to market: Phillip Sharp is one of several RNAi researchers to form start-up biotech firms.

system were activated, a mechanism by which cells shut themselves down in response to invading germs. The siRNAs activated genes that act early in the interferon pathway, and Williams' group did not measure whether the activated genes stopped working. But the team says that its findings provide a warning that off-target effects are perhaps more common than scientists have realized.

Researchers argue that these hints of off-target RNAi effects highlight the need for a deeper understanding of how, exactly, the system works. For instance, we still don't know for sure how many proteins work together to shut down a target mRNA. It's also unclear why some siRNAs are incredibly effective, whereas others, targeted at a different region of the same gene, don't work as well. Given these unknowns, some researchers urge caution before rushing into clinical trials. "Before you know what you could perturb, you have to know what's there," says Tom Tuschl, a biochemist and RNAi pioneer at Rockefeller University in New York.

Even some of the scientists working in the commercial sector, where excitement about the clinical prospects of RNAi is most intense, agree that a great deal of groundwork remains to be done. "For real clinical development, this has to be done right," says Khvorova. "Investing a little more time on the basic steps will pay back in years of time saved later on."

But despite all of these caveats, most researchers working in this fast-moving field have high hopes that RNAi will deliver on its therapeutic promise. "This is the honeymoon period; things are looking great," says Kay. "We will encounter technological issues along the way, but our goal is to solve these problems and get it to work." ∎

Erika Check is *Nature*'s Washington biomedical correspondent.
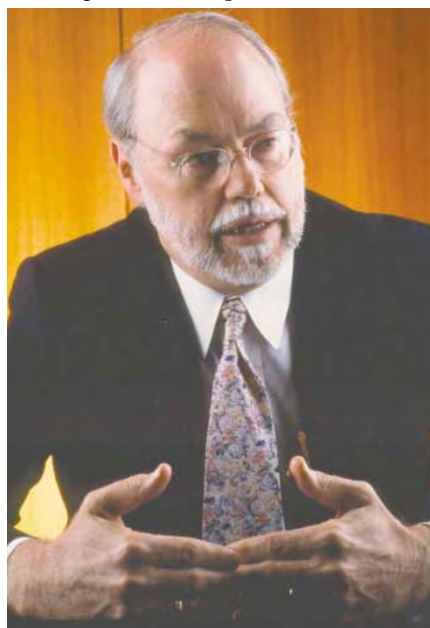
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JOINT APPENDIX 34

New structures in complex formation between DNA and cationic liposomes visualized by freeze–fracture electron microscopy

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Abstract Structures formed during interaction of cationic liposomes and plasmid DNA were studied by freeze–fracture electron microscopy and their morphology was found to be dependent on incubation time and DNA concentration. These structures were formed with liposomes composed of DC-Chol and DOPE after 30 min incubation at DNA : lipid concentrations encompassing maximal transfection activity. They resembled liposome complexes (meatballs) and additionally bilayer-covered DNA tubules (spaghetti), whereby the DNA-tubules were found to be connected to the liposome complexes as well as occurring free in the suspension. At later times and higher DNA-to-liposome ratios the complexes grow larger while their membranes become discontinuous, allowing the self-encapsulation of the DNA. The relative transfection potency of the various morphologically distinct structures is discussed.

Key words: Cationic liposome; Plasmid DNA; Transfection; Freeze–fracture electron microscopy

1. Introduction

Gene therapy and genetic engineering require reliable and efficient systems for delivery of exogenous genes into target cells. Among other non-viral vectors, liposomes have been widely used as delivery agents for DNA and other polynucleotides (for reviews see [1,2]). Thereby, liposomes offer several advantages over viral vectors, including the absence of viral components, the protection of the DNA/RNA from inactivation or degradation, and the possibility for cell-specific targeting. Negatively charged liposomes, used for gene delivery in vitro to mammalian cells [3,4] or plant protoplast, however, do not deliver more effectively than other simpler methods, despite various improvements such as pH-sensitive liposomes [5] or virosomes [6].

An important breakthrough in this field was the design of positively-charged liposomes, or cationic liposomes, as transfection agents [7–14]. Since nucleic acids are highly negatively charged molecules they can interact spontaneously with the cationic liposomes. Complete complexation is achieved (even at low liposome-to-DNA ratios) simply by mixing the polynucleotides with preformed cationic liposomes. This simple and mild but still effective method is based on a complex formation between plasmid DNA and vesicles composed of synthetic cationic surfactants such as DOTMA [7], or DC-Chol [12] in combination with a helper lipid, e.g. DOPE [2,7,9,11,12,14]. Most of the cationic surfactants form micelles but not liposomes, and the addition of a phospholipid having a strong tendency to adopt the inverted hexagonal structure (H_{II} phase), such as DOPE, was found to be important [2]. DOPE helps in

forming liposomes with a significantly increased transfection efficiency [15] while it reduces the cytotoxicity of the cationic surfactants [13]. A variety of different quaternary ammonium surfactants with alkyl-, ether- and ester-links to various backbones have been investigated in the search for an optimal transfection agent that is less toxic and metabolized by the cells [9–12]. For a given cell confluence, DC-Chol liposomes, for instance, have been found to be at least fivefold less toxic than that of lipofectin, which contains DOTMA [12]. More recently, cationic liposome–DNA complexes have been used successfully to express heterologous genes in vivo, after administration intravenously [16], intra-arterially [17], intra-tracheally [16], or by aerosol inhalation [18].

Despite their extensive use as transfection agents the mechanism of DNA interacting with cationic liposomes and the structure of the resulting complexes are still poorly understood. It is generally assumed that, in contrast to the negatively charged liposomes, there is no true encapsulation of the DNA by the cationic vesicles, but rather binding at their surface while the size and shape of the vesicle are maintained [8]. Electron micrographs of a recent study [19] suggest that the cationic liposomes are attached like beads on a string, gradually covering the DNA chain until, at a certain lipid to DNA ratio, a complete lipid coating of the DNA is reached composed of multilayered liposomes. It seems that this lipid coating is able to protect the DNA, to enhance the uptake by recipient cells, possibly via endocytosis and/or fusion, and possibly also to deliver material into the nucleus [20].

In this present study, we have investigated the structure of the complexes formed between plasmid DNA and preformed cationic liposomes composed of DC-Chol/DOPE, by freeze–fracture electron microscopy, and looked for the structural modifications of the complexes and their dependence on varying DNA concentration and incubation time. Although naked DNA is not visible by freeze–fracture electron microscopy, this technique is very useful for studying the interaction between DNA and cationic liposomes because the structure of the DNA is enhanced during this process by lipid coating, probably consisting of a single bilayer tubule.

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Abbreviations: DC-Chol, $3\beta[N-(N',N'-dimethylaminoet hane)-carbamoyl]$cholesterol; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOTMA, N-[1-[2,3-dioleyloxy)propyl]-*n,n,n* -trimethylammonium chloride.

2. Materials and methods

2.1. DNA, surfactant, lipid, and preparation of the liposomes

Plasmid pRSV-LUC, containing a luciferase gene driven by the Rouse sarcoma virus promoter [21] was prepared by a CsCl/EtBr gradient method [22]. The plasmid DNA was of the B-type (2.37 nm) and existed predominantly in the supercoiled state. DOPE was purchased from Avanti Polar Lipids Inc. DC-Chol was synthesized according to the method of Gao and Huang [12]. DC-Chol and DOPE were mixed in a 3:2 molar ratio, evaporated in a stream of nitrogen to form a thin film, and vacuum desiccated for several hours to remove any residual chloroform. The lipid film was rehydrated in distilled deionized water which had been autoclaved. The liposomes were allowed to hydrate overnight. DC-Chol liposomes were prepared by microfluidization using a M-110S microfluidizer (Microfluidics Corp.) to an average diameter below 200 nm. The particle size was measured by using a Coulter sub-micron particle analyzer, using a uni-modal analysis. The sample was counted for 200 s. The liposomes were filtered through a $0.2 \mu m$ filter to provide sterilization. The liposomes were diluted to a final concentration of 2 μmol/ml (1.2 mg/ml of total lipid).

2.2. Formation of the DNA–liposome complexes

To a volume of 10 μl HEPES buffer (20 mM, pH 7.5) 20 μl of DC-Chol liposomes (2 μmol/ml) with varying amounts of pRSV-LUC (1 mg/ml) were mixed at room temperature by a Hamilton syringe, to obtain final DNA-to-lipid ratio ranging from 1–10 μg of DNA to 20 nmol DC-Chol liposomes. Thereby, the total volume of the sample was increased, resulting in concentrations ranging from 31.25–200 μg of DNA/ml. The concentration of the suspensions, chosen for freeze–fracture electron microscopy was approximately 15- to 100-fold-higher than what is used in transfection because it is always a problem to refine tiny little structures such as interaction events in too dilute suspensions at the microscope.

DNA–DC-Chol complexes were investigated by freeze–fracture electron microscopy at room temperature, after different incubation times, ranging from 10 min to 24 h, and at various DNA-to-lipid ratios, ranging from 1–10 μg of DNA per 20 nmol DC-Chol liposomes.

2.3. Freeze–fracture electron microscopy

DC-Chol liposomes, naked DNA, and DNA–liposome complexes were quenched rapidly for freeze–fracture electron microscopy using the sandwich technique and liquid propane (cooling rate $>10^4$ K/s). The cryofixed specimens were fractured and shadowed in a Balzers BAF 400D freeze–fracture device at -120°C and 2×10^{-6} Torr. The cleaned replicas were examined in a transmission electron microscope (Jeol JEM 100B or Zeiss CEM 902 A) [23].

3. Results and discussion

We have investigated the complex formation between plasmid DNA and preformed cationic liposomes by freeze–fracture electron microscopy and studied the structural modifications of both components, depending on DNA concentration- and incubation time.

3.1. Liposome control

Freeze–fracture electron micrographs of liposomes, made of DC-Chol/DOPE (6:4 by molar ratio; 2 μmol lipid/ml; 'liposome control') and prepared by microfluidization, showed well-defined, mainly small liposomes which were well separated from each other in the ice (Fig. 1A). The liposomes were fairly uniform in size, showing mean diameters below 200 nm, which is in good agreement with laser light scattering measurements. Due to the surface charge on the liposomes, contributed by the positively charged DC-Chol component, there is a repulsive force between the liposomes, preventing aggregation.

3.2. DNA control

In freeze–fracture electron micrographs of the pRSV-LUC DNA (0.67 mg DNA per ml HEPES buffer, 20 mM, pH 7.5; 'DNA control') it was not possible to clearly visualize the naked plasmid DNA (Fig. 1B, some very weak features are marked by an arrow head). With a maximal width of 2.37 nm of the DNA strands, these structures are at the resolution limit of freeze–fracture technique (about 2 nm for periodical structures [23]). This is also true of another cryo-technique, cryo-electron microscopy, where some effort is needed to visualize the structure of naked DNA in detail [24]. Typically, DNA samples for electron microscopy are prepared by the Kleinschmidt method of DNA spreading followed by metal rotary shadowing [25]. One drawback of this method in visualization of the DNA together with liposomes is that the Kleinschmidt methodology does not allow a size comparison of these species, since DNA molecules are detected as cytochrome c–DNA complexes and appear much bigger than their naked form [19].

3.3. DNA–liposome complexes

Freeze–fracture electron micrographs of DNA–liposome complexes, made after a short incubation time (10 min, Fig. 1C, left panel) or low DNA-to-lipid ratio (1 μg/20 nmol DC-Chol, Fig. 1C, right panel), showed semi-fused liposomes where the number of liposomes involved was low (mainly 2–3 liposomes, Fig. 1C) and their size was roughly the same compared to the control liposomes as shown in Fig. 1A. As described in the figure legends, the bars represent 100 nm for all the electron micrographs. The reason for choosing a 2-fold (in Fig. 1C, left panel) and a 3-fold higher magnification (in Fig. 1C, right panel) compared to Fig. 1A is to demonstrate possible fusion structures. Indeed, in Fig. 1C (left panel), lipid particles (marked by arrows) were visible at the fusion area. The negatively charged plasmid DNA (not visible) seems to act as a fusogenic agent, drawing together the positively charged liposomes and forming semi-fused liposomes (liposome–DNA aggregates, Fig. 2).

At longer incubation times (30 min–24 h) and at higher DNA-to-lipid ratios (2–10 μg of DNA to 20 nmol of DC-Chol liposomes), some proportions of the DNA clearly became visible, as shown in Fig. 1D–G and Fig. 2. Obviously, its structure was enhanced, presumably by lipid-coating. The diameter of these tubular spaghetti-like structures (Fig. 1D; some of the

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Fig. 1. Freeze–fracture electron micrographs of (A) liposomes, made of DC-Chol/DOPE (6:4 by mol; liposome control); (B) naked pRSV-Leu DNA (DNA control, some of the very weak features are marked by an arrow head); and (C–G) DC-Chol–DNA complexes, at various DNA-to-lipid ratios and incubation times: (C) fused liposomes without spaghetti-like structures at 10 min incubation time and 4 μg DNA/20 nmol DC-Chol in the left panel and at 24 h incubation time and 1 μg DNA/20 nmol DC-Chol in the right panel (some lipidic particles are marked by arrows); (D) spaghetti–meatball complexes at 24 h incubation time and 2 μg DNA/20 nmol DC-Chol (some spaghetti, connected with the liposomes, are marked by an arrow); (E) 'free' spaghetti-like structures, attached to the metal foil of the sandwich at the same incubation time and concentration as in D; (F) spaghetti–meatball assembly at 30 min incubation time and 4 μg DNA/20 nmol DC-Chol, and (G) spaghetti–meatball assembly with partly disrupted liposome membranes at the same DNA-to-lipid ratio as in F but at 60 min incubation time. The bar on all electron micrographs represents 100 nm and the shadow direction is running from bottom to top.

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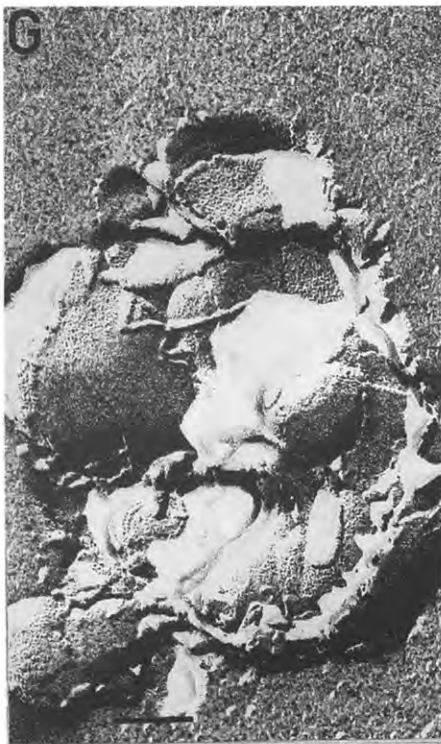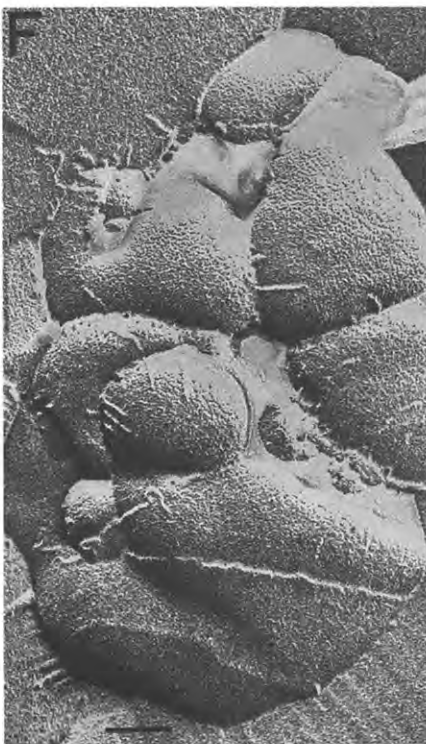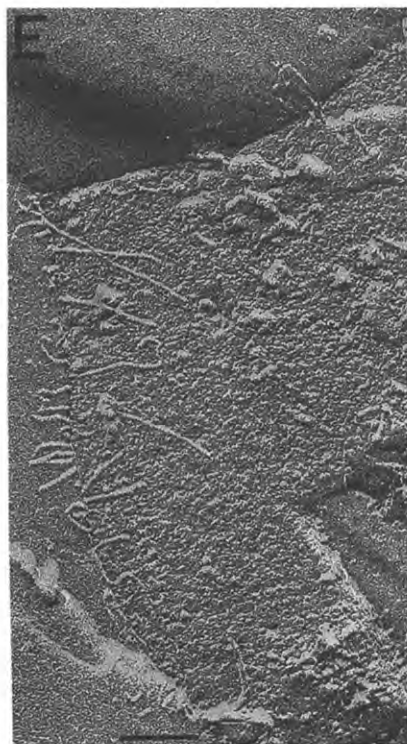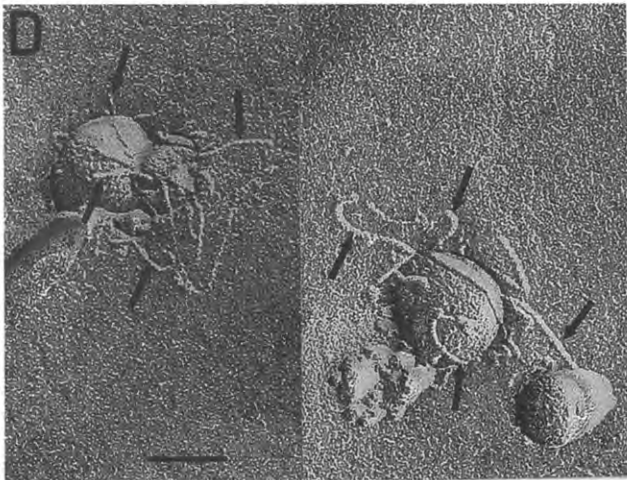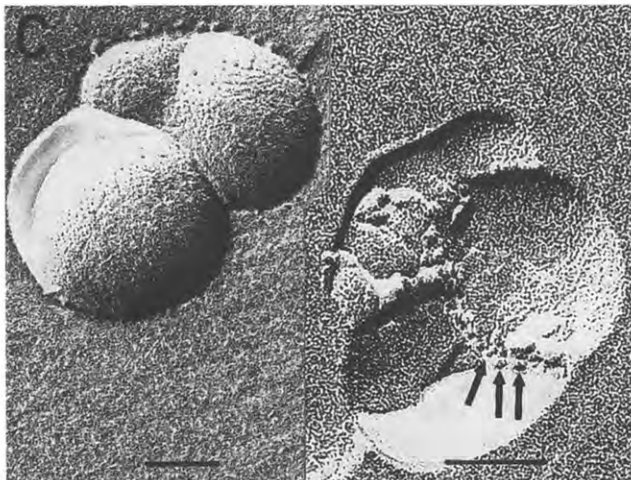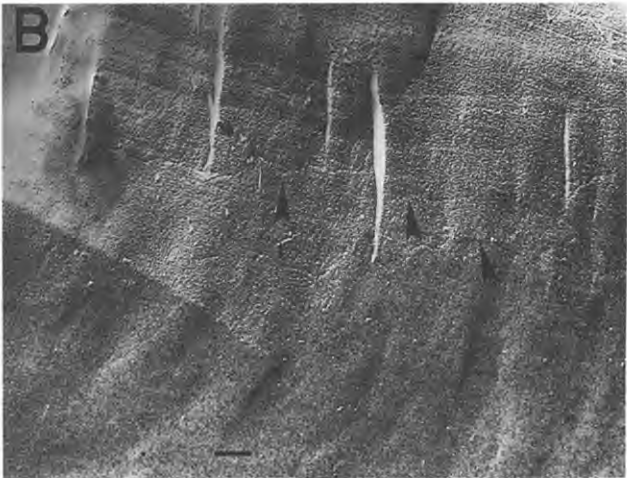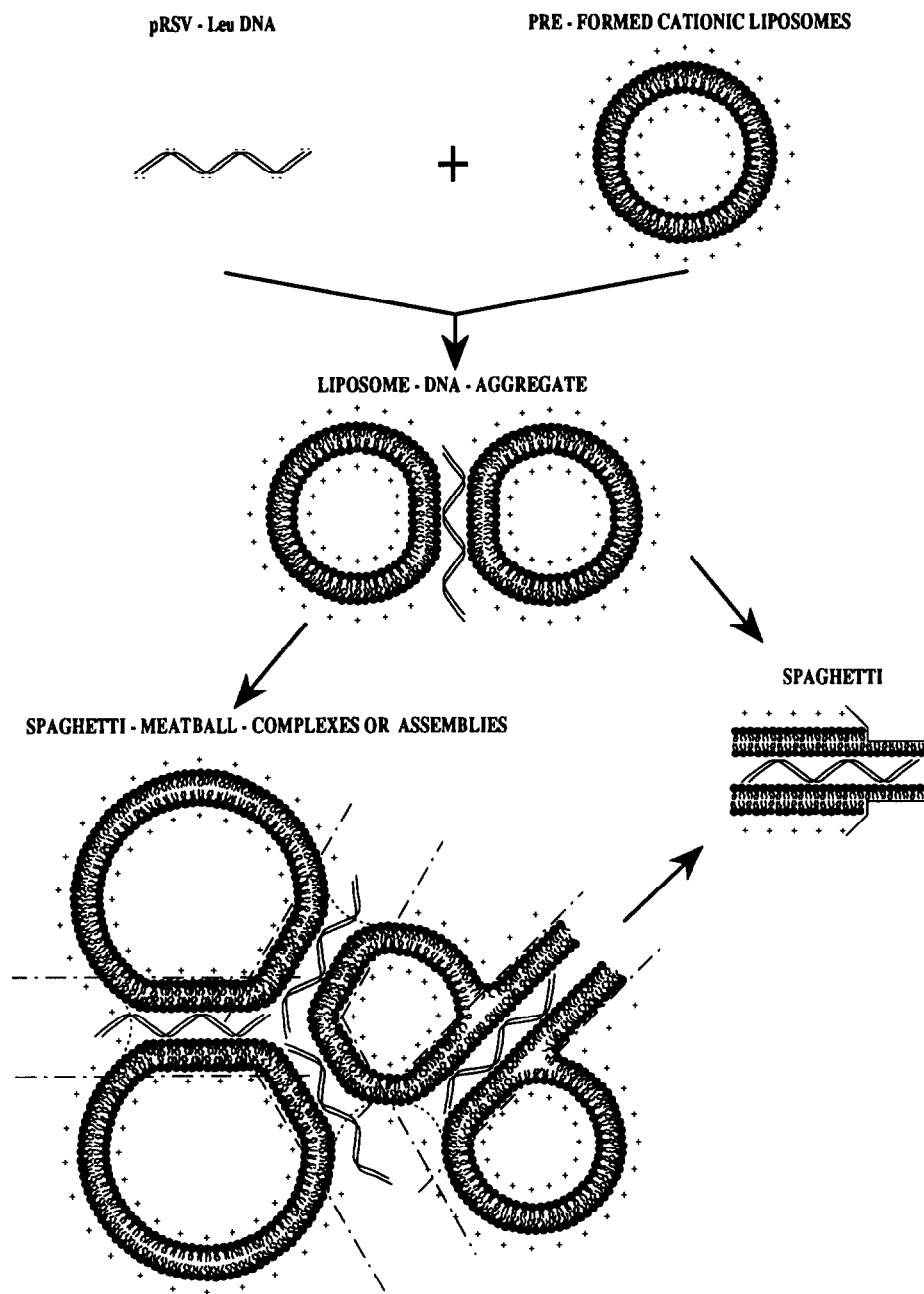
Fig. 2. Scheme showing our personal suggestion, based on freeze–fracture electron micrographs, about the interaction of negatively charged DNA with the cationic DC-Chol-containing liposomes and the formation of liposome–DNA aggregates without spaghetti-like structures, occurring at short incubation times and low DNA-to-lipid ratios, and spaghetti–meatball complexes, spaghetti–meatball assembles and spaghetti-like structures, connected with the semi-fused liposomes but also occurring 'free' in suspension at longer incubation times and higher DNA-to-lipid ratios.

spaghetti are marked by arrows) was approximately 7 nm. Both convex DNA strands (shadow behind) as well as concave DNA furrows (shadow in front, one of them is marked by an arrow in Fig. 3A) are found. These findings support the possibility of a bilayer tubule covering the DNA strands. During freeze–fracturing, the fracture plane usually follows the hydrophobic interior of a bilayer, whether it is surrounding a cell or a liposome [26], or in this case, while surrounding a DNA strand. This produces a theoretical diameter of 6.37 nm for the spaghetti-like structure by the summing of 2 nm of each of the two fluid bilayer halves and 2.37 nm of the diameter of the plasmid

DNA (Fig. 3B). Measured and theoretical diameters of the spaghetti are in good agreement and support the hypothesis of a single bilayer tube coating the DNA.

The strong charge interaction between the positively charged DC-Chol and the negatively charged DNA may stabilize the high curvature of the bilayer tubule around the DNA strand, and the high content of DOPE may also assist in the stabilization of the spaghetti structure. Based on its wedge-shaped molecular structure, DOPE can adopt highly curved structures such as H_{II} tubules, at excessive lipid and/or high temperatures [27], or other non-bilayer structures [28]. Freeze–fracture elec-

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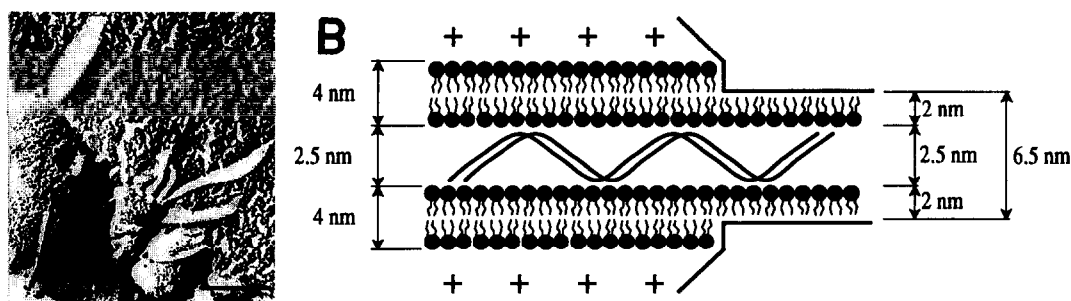
Fig. 3. (A) Cut-out of the freeze–fracture electron micrograph of Fig. 1F showing enlarged convex DNA strands and a concave DNA furrow, marked by an arrow. The bar represents 100 nm and the shadow direction is running from bottom to top. (B) Cross-fraction of a spaghetti.

tron microscopy is one of the best methods of distinguishing between bilayer and non-bilayer structures [23] and to visualize hexagonal tubules [23,28]. However, no hexagonal tubules were visible at any time in our investigation of DNA–cationic liposome systems, containing excess water, as observed in DNA–lipid systems with excess lipid [29].

Spaghetti-like structures were found to be still connected to the liposomes (spaghetti–meatball complex, Fig. 1D; spaghetti–meatball assemblies, Fig. 1F,G; scheme of Fig. 2), but were also found separated from the liposomes, 'free' in suspension (Figs. 1E and 2). Possibly due to the residual positive charge at their surfaces, spaghetti-like structures are often found in contact with the metal foil of the sandwich which was used for the rapid freezing of the samples (Fig. 1E).

With higher DNA concentrations and longer incubation times (beginning with 4 μg of DNA per 20 nmol DC-Chol liposomes at 30 min) a proportion of the DNA–liposome complexes were observed as larger structures containing several semi- or totally fused liposomes (spaghetti–meatball assemblies; Figs. 1F,G and 2). Most of the liposomes involved in the spaghetti–meatball assemblies had grown in size in comparison to the control liposomes (Fig. 1A), presumably by fusion of these liposomes (Figs. 1F,G and 2). During the formation and growth in size of the spaghetti–meatball assemblies the membranes of the liposomes appeared discontinuous, allowing for self-encapsulation of the DNA (Fig. 1G). All distinct structures, such as spaghetti–meatball complexes, spaghetti–meatball assemblies, as well as 'free' spaghetti, were found at incubation times from 30 min to 24 h and at DNA-to-lipid ratios from 2 to 10 μg of DNA per 20 nmol of liposomes. However, the proportion of different structures were different. With increasing DNA-to-lipid ratios, the content of spaghetti–meatball assemblies increased whereas the content of spaghetti–meatball complexes decreased.

From an examination of the optimal DNA-to-lipid ratio for transfection and their dimension, it seems likely that the spaghetti-like structures may be the active DNA–lipid complex. The spaghetti-like structures occur at DNA:lipid concentrations which are typically used during transfection (2 μg of DNA to 20 nmol DC-Chol liposomes) and their diameter comes closest to the diameter of the nuclear pores [30]. However, calculating more precisely the diameter of the DNA–lipid tubules surrounded by one full bilayer, which is about 10 nm (Fig. 3B), and the diameter of the nuclear pores (7 nm; [30]) even the spaghetti are too thick to pass these pores freely. On the other hand, spaghetti, similar to microvilli, are extremely curved structures

with very small radii (especially at their ending tips) and therefore are able to adhere and fuse to flat cells easily [31]. Additionally, spaghetti-like structures as well as spaghetti–meatball complexes and assemblies, which were observed during our investigation, may still bear residual positive charges on their surfaces. This may also lead to an interaction and fusion with cell- and probably nuclear membranes, thereby promoting the transfer of the DNA into the cytoplasm and eventually into the nucleus of the cells.

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JOINT APPENDIX 35

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Lipid nanoparticles for nucleic acid delivery: Current perspectives

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ABSTRACT

Nucleic Acid (NA) based therapeutics are poised to disrupt modern medicine and augment traditional pharmaceutics in a meaningful way. However, a key challenge to advancing NA therapies into the clinical setting and on to the market is the safe and effective delivery to the target tissue and cell. Lipid Nanoparticles (LNP) have been extensively investigated and are currently the most advanced vector for the delivery of NA drugs, as evidenced by the approval of Onpattro for treatment of Amyloidosis in the US and EU in 2018. This article provides a comprehensive review of the state-of-the-art for LNP technology. We discuss key advances in the design and development of LNP, leading to a broad range of therapeutic applications. Finally, the current status of this technology in clinical trials and its future prospects are discussed.

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1. Introduction

Lipid Nanoparticles (LNP) have been extensively investigated for the delivery of nucleic acid (NA) drugs, culminating with the approval of Onpattro for treatment of Amyloidosis in the US and EU in 2018. This has provided strong validation for the use of LNP for systemic siRNA delivery, and LNP are increasingly being utilized in the burgeoning fields of mRNA and replicon-based therapeutics – in oncology and infectious disease vaccine contexts, for therapeutic protein replacement strategies, and to enable gene editing approaches. These payloads are larger, more fragile and potentially more immunogenic than their oligonucleotide counterparts. They do not tolerate 2'-hydroxy chemical modification to the same degree, and as such the delivery challenge is even more acute. A wide range of LNP have been used for these purposes by many groups, but general principles have emerged regarding the properties of well-tolerated, potent LNP (and the NA "payload"). In particular, LNP for NA delivery have distinct, critical lipid components and proportions, specialized manufacturing and control techniques, and complex morphology. This paper reviews the historical development of LNP design for a variety of NA payloads and surveys the current clinical trial landscape for this class of pharmaceuticals.

2. Lipid nanoparticles: overview, composition, formation mechanism

The advanced NA LNP delivery systems of today have evolved away from both liposomal systems and lipoplexes to become more efficient and effective delivery vehicles of NA. Indeed, the LNP used today for NA delivery are quite different from classical liposomes. Perhaps one of the most distinctive differences lies in the fact that LNP do not display a lipid bilayer surrounding an aqueous core (Fig. 1A). Additionally, the current-day LNP do not form particles with nucleic acid payloads driven by electrostatic complexation, nor do they need to balance the charges of constituent compounds for effective and efficient delivery into a cell.

The first reports of some NA encapsulation in liposomes originated in the late 1970's [1–3]; these systems suffered from poor encapsulation, in part due to the use of neutral lipids and passive encapsulation procedures. Similarly, lipoplexes have been extensively used *in vitro* for preclinical experimentation but have shown potency and tolerability issues, thereby precluding their use in the clinic. The poor potency and tolerability of lipoplexes is in part due to the use of constitutively charged cationic lipids that complex with the negatively charged NA. When administered intravenously, this leads to rapid elimination from the blood and activation of the immune system due to the charged nature, propensity to aggregate, and unencapsulated, exposed position of the NA. Permanently-charged cationic lipids have also been employed in earlier lipid formulations, leading to higher efficiency of NA encapsulation than in neutral systems, due to the charge interactions between lipid and NA. Still, these formulations suffered from poor pharmaceutics and tolerability due to the introduction of a highly charged system into the blood. A breakthrough in the development of pharmacologically acceptable lipid formulations was the advent of ionizable cationic lipids [4]. These pH-titratable lipids are positively charged at acidic pH but mostly uncharged at the pH of blood. This feature confers on the lipid/LNP the advantages of positive charge (the ability to complex NA and

the ability to interact with the endosomal membrane as part of the fusion process to release NA into the cytosol of the cell), without the drawbacks (rapid elimination and poor tolerability). These titratable aminolipids are most often combined with PEGylated lipids, which aid in the formulation process leading to nanometer-scale structures (Fig. 1C), increase vial stability and help to mask the surface of the LNP from blood components to optimize the pharmacokinetics and biodistribution of the product. PEGylation, however, interferes with cellular uptake via endocytosis. Therefore, a critical design iteration that contributed to the success of the current generation of LNP is the use of PEGylated lipids that diffuse out of the LNP on administration and enable cellular uptake.

2.1. Ionizable cationic lipids

The ionizable lipid is a critical component of LNP, and a major determinant of LNP potency. Their use for nucleic acid delivery was an important divergence from the permanently charged cationic lipids used previously. First reported for NA delivery by Semple et al. [4], the ionizable nature promotes the formation of particles with an encapsulated payload, as opposed to complexation. The lack of substantial positive charge at physiological pH leads to improved pharmacokinetics, since the particles are not cleared rapidly by the reticuloendothelial system (RES). This yields an appreciable half-life in the bloodstream (30 minutes or greater, depending on other components used), permitting better accumulation in target tissues, such as liver and solid tumours. The ionizable nature further improves the tolerability profile, since permanently charged cationic lipids are prone to causing cellular toxicity, and particles that contain them are more likely to stimulate the immune system and/or aggregate in the blood, so depositing in fine capillary beds such as the lung.

There are many facets to the optimal design of ionizable lipids, and following Semple et al.'s [4] disclosure of DODAP, several groups began to explore this area. The next key advance was the discovery that increasing the degree of unsaturation in the hydrophobic domain of the ionizable lipid would dramatically increase potency [5]. DODAP, like many cationic lipids used to that point, employed oleyl groups – a C_{18} carbon chain with a single *cis*-double bond. The double bond induces kinks in the chain, and Heyes et al. [5] showed that increasing the number of double bonds more readily promoted the formation of the fusogenic, H_{II} hexagonal phase in the particle. By increasing the number of double bonds to 2 in close proximity (linoleyl group), endosomal fusion and delivery were improved substantially. The resulting lipid DLinDMA enabled the first demonstration of RNAi in primates with a systemically administered LNP [6], and became the first ionizable lipid to enter human clinical trials in an siRNA-LNP.

Using DLinDMA as a starting point, Semple et al. embarked on a new SAR study, publishing results that described several more key features in ionizable lipid design, and the LNP platform in general [7]. First, the means of connecting the linoleyl chains to the rest of the lipid was highlighted. Esters, carbamates and thioethers were all found to be inferior to DLinDMA's ethers. However, a ketal ring, with a single carbon atom as the point of attachment for both chains, yielded a considerably improved potency. In the same study, the authors noted that the apparent acid dissociation constant (pKa) of the LNP could be adjusted by
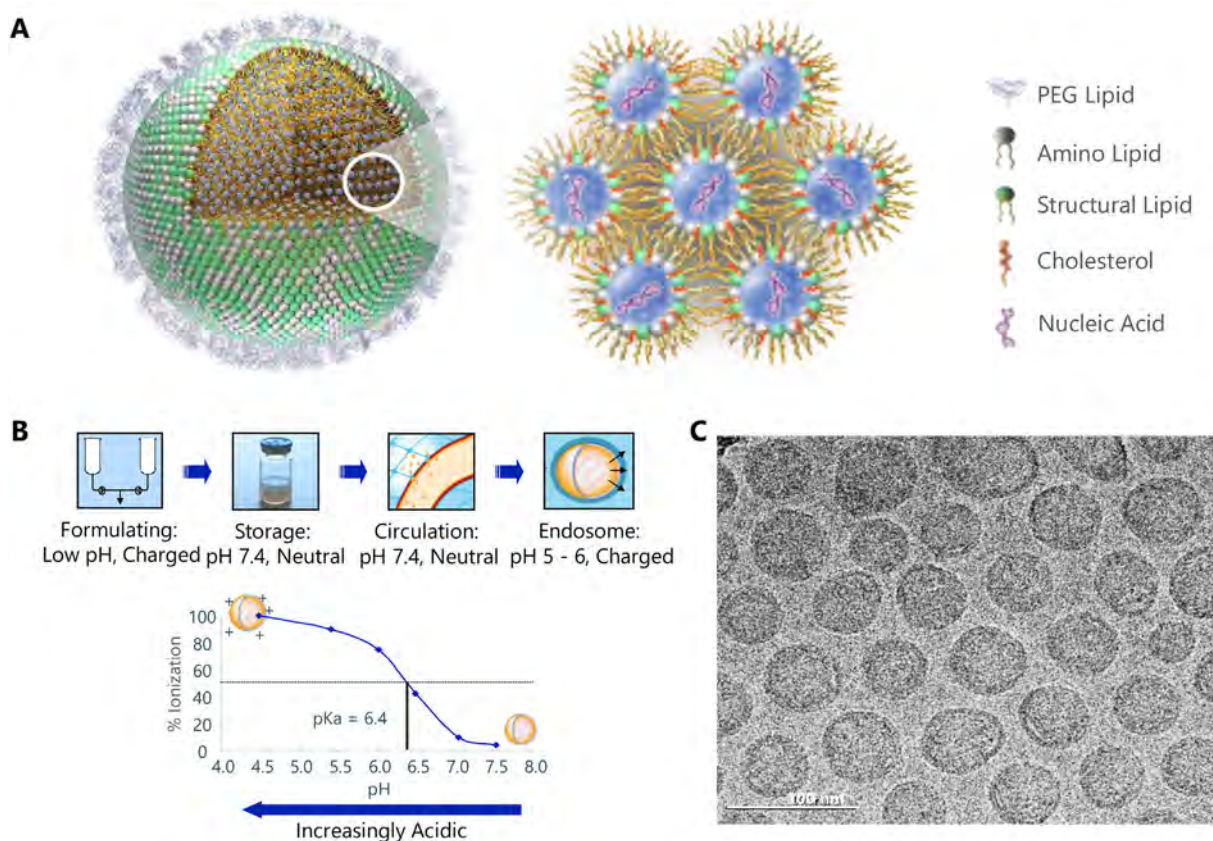
Fig. 1. (A) Schematic representation of Lipid nanoparticle (LNP) structure. (B) Principle and importance of pKa in the action mechanism of ionizable lipids. (C) Cryogenic electron micrograph of LNPs at a magnification of 110,000x. Scale Bar: 100 nm.

changing the length of the carbon spacer between the ionizable lipid's head group and the rest of the molecule. The pKa is essentially a measure of the pH at which ionizable lipid/LNP becomes positively charged, and it was shown to have a strong effect on potency; a series of ketal analogs that differed only in the length of their carbon spacer (1 to 4 carbons) differed by more than 30-fold in potency. At the time, the best performing lipid was DLin-KC2-DMA (KC2 – Fig. 2). The precise pKa of the LNP is important since the particle's surface must be essentially neutral at physiological pH (i.e. in the bloodstream) but become charged inside the acidifying endosome after cellular uptake (Fig. 1B). Lastly, but equally important, were the changes made to the LNP composition. By reducing the PEG content from 10% to just 1.4 mol% and markedly increasing the ionizable lipid content to 57%, the authors gained a further 5-fold increase in potency without changing the lipid components [7]. Similar compositions are still used today, including Onpattro™ and many mRNA-based compositions currently being evaluated in the clinic, which have molar ratios of 1.5% PEG-lipid and 50% ionizable lipid (Table 2).

These structural features were further optimized in the elucidation of DLin-MC3-DMA (MC3 – Fig. 2). In yet another illuminating SAR screen, Jayaraman et al. demonstrated very clearly that the pKa should be between 6.2 and 6.5 for optimum activity in the hepatocyte [8]. They further showed that by maintaining KC2's single carbon point of attachment for the linoleyl chains, but exchanging the ketal linker for a carboxylic ester, further gains to potency could be realized. The resulting lipid, MC3, is the ionizable lipid used in the Onpattro product [8,9].

Several groups have described "biodegradable" LNP, which comprise enzymatically-labile ionizable lipids [10,11]. MC3 is not biodegradable and has a serum half-life of approximately 70h [12]; it has proven safe and well-tolerated at doses of 0.3 mg kg^{-1} every three weeks in Onpattro™, with no evidence of lipid-mediated toxicity. Biodegradability is potentially more important for LNP applications that require more frequent administration, for example mRNA payloads encoding a therapeutic protein. Precise dosing frequency will be determined by the rate of transcript turnover and protein product half-life the protein product – but early reports indicate that for some important disease targets the dosing interval will be more frequent than siRNA [11,13]. As a major component of most LNP products, ionizable lipids should be considered first for redesigning with biodegradable properties. It is most often built into the lipid by means of carboxylic ester groups which can be cleaved by esterases [14]. The strategy is effective, and these compounds are metabolized and cleared in a matter of hours, although the precise location of the carboxylic ester moiety is of utmost importance. L319 (Fig. 2) shows rapid degradation in vivo, whereas the closely related L343 (Fig. 2) shows extended stability, presumed to be due to steric hindrance protecting the ester from the action of the enzyme. Such biodegradable lipids have been reported to be even more potent than MC3, with the compounds described by Sabnis et al. [11] furnishing as much as 5-fold more protein expression.

A subset of ionizable lipids called «lipidoids» were first reported in 2008 by Akinc et al. [15]. These dendrimer-like structures were designed to be synthesized in a short number of steps, using straightforward chemistry that would easily lend itself to a high throughput approach. In this way, large libraries of hundreds of compounds could quickly be made to study structure activity relationships. The first lipidoid of note, 98N12-5 (aka ND98), was used to deliver siRNA to hepatocytes [15] and endothelial cells [16], and required relatively high doses. But like canonical ionizable lipids, later generations of lipidoids have improved in potency and general performance considerably, with examples such as C12-200, cKK-E12, OF-02 and 503-O13 being used at much lower doses [17–20]. Interestingly, they have been
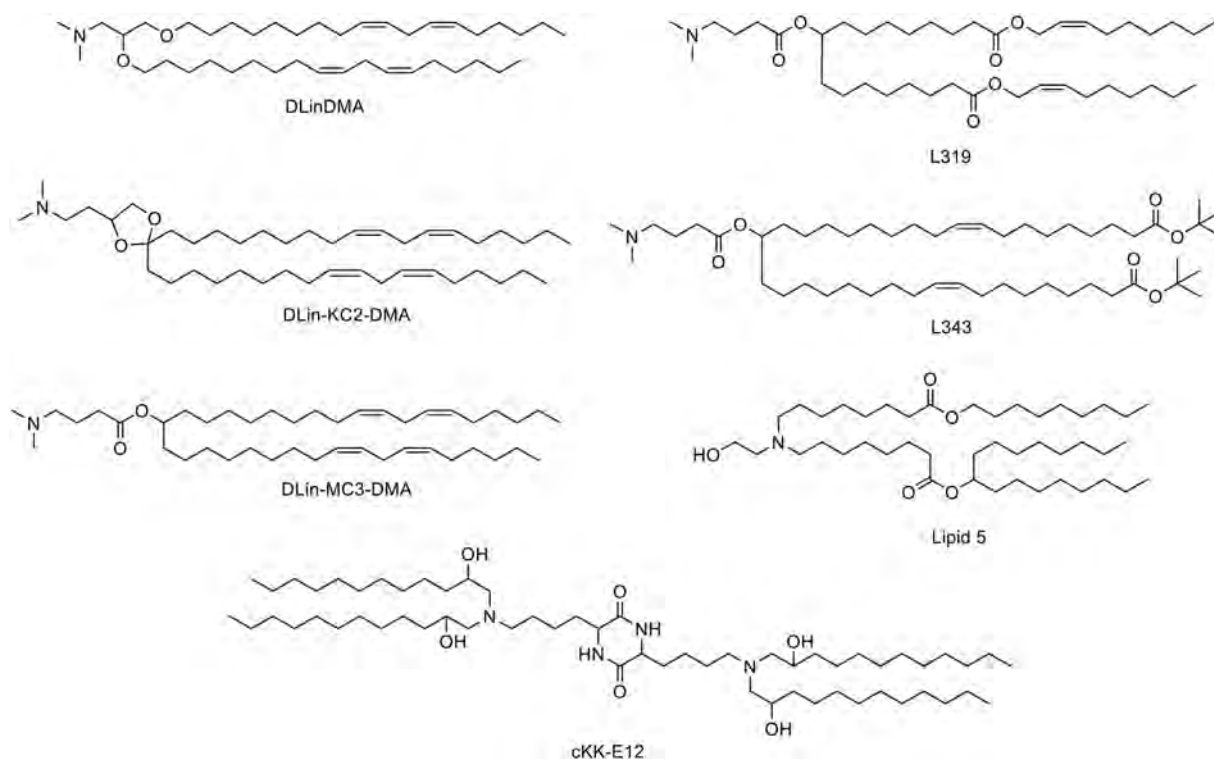
Fig. 2. Ionizable lipids of note.

shown to be less dependent on ApoE for uptake, and screening suggests that they are best suited to formulating with DOPE, rather than DSPC like other ionizable lipids [21].

2.2. PEGylated lipid

PEG-lipids are another important LNP component that plays several roles. Their structure consists of two domains: a hydrophilic PEG-polymer conjugated to a hydrophobic lipid anchor. They are situated at the surface of lipid particles, with the lipid domain buried down into the particle and the PEG domain extending out from the surface. Historically, they were used in liposomal systems to extend circulation time, since the PEG polymer represents a steric barrier preventing binding of plasma proteins (opsonins) that would otherwise result in their rapid clearance by the reticuloendothelial system. A longer residence time in the blood meant particles were more likely to accumulate in disease sites like solid tumours, due to the enhanced permeation and retention effect (EPR). They were used for similar reasons in stabilized plasmid–lipid particles (SPLP), an earlier iteration of LNP that delivered plasmid DNA for gene therapy applications [22–24].

In present day LNP however, PEG-lipids have been further designed to balance circulation time and cellular uptake, as well as appropriately determine the particle size during manufacturing. Without a PEG-lipid present, the low pH and ethanolic environment of the LNP manufacturing process would promote particle fusion as the particles form. The steric barrier of the PEG-lipid prevents this and helps yield a homogenous particle population with narrow polydispersity and small particle size – typically 50 – 100 nm, depending on the exact amount of PEG-lipid used.

As with older lipid particle applications, PEG-lipids are helpful in preventing unwanted opsonization in the blood, but they also interfere with important steps associated with successful payload delivery and activity. First, LNP are well suited and often used for delivery to hepatocytes - and the mechanism of uptake involves ApoE binding to the LNP surface in the bloodstream [15]. A fully intact PEG surface layer

interferes with this step. Second, the endosomal escape, which must occur for successful intracellular delivery of the NA payload, is impeded as the PEG layer prevents the close approach and fusion of the LNP with the endosomal bilayer [25]. For these reasons, the PEG content is kept to a minimum, and these concepts (along with increased ionizable content) underpin the 5-fold potency improvement reported by Semple et al. when decreasing the PEG content of the formulation from 10% to ~ 1.5 mol% [7].

PEG-lipids can be engineered to diffuse away from the particle, and given the above, the rate at which they do so is clearly important. This rate can be adjusted by changing the size of the PEG-lipid's anchor, a feature that was originally used in liposomal systems in an oncology setting to produce particles that circulated for a longer duration in the blood [22,23,26]. As outlined above, however, for hepatocytes, a shorter residence time is favored and thus smaller lipid anchors are used – two C_{14} chains have been found optimal and commonly used since the earliest days of in vivo siRNA-LNP delivery [27]. PEG-lipid desorption rates were characterized in more detail by Mui et al, who used dual labeled LNP containing ^{14}C-MC3 and ^{3}H-PEG-lipid to measure and correlate to pharmacokinetics and biodistribution.

PEG diffusion rates are also important for safety reasons. Permanently bound PEG-lipids (and the resulting longer circulating particles) have been found to promote immunogenicity when used in conjunction with nucleic acid payloads. A surprisingly robust, long-lived antibody response is generated against the surface chemistry (i.e. PEG) of such particles, resulting in accelerated clearance from the blood and acute hypersensitivity upon repeat administration [28,29]. This is discussed in more detail in a later section.

2.3. Phospholipids and cholesterol

Since the inception of LNP for siRNA and their more recent application to mRNA, LNP have employed phospholipids and cholesterol for their helpful contributions to the structural integrity and phase transition behavior of the LNP. This in turn influences the fusogenicity of the

particles. They are required to ensure appropriate encapsulation of the NA payload and stability over time. Additionally, the presence of phospholipid aids in the work up of formulation via tangential flow ultrafiltration (TFU). Some studies have shown that the identity of phospholipid can have a strong impact on potency [21], although the outcome may depend on other lipids being used in the system.

A recently published SAR study on analogs of cholesterol showed that incorporation of C-24 alkyl phytosterols into LNP enhanced gene transfection considerably [30]. The length of alkyl tail, flexibility of sterol ring and polarity due to -OH group were all found to be important. The studies were performed in vitro.

2.4. Nucleic acid

Without a doubt, the development of LNP, with input from a number of key research groups, has been critical in launching and realizing the era of RNA interference. In the clinic, they have been used to deliver siRNA and generate proof of concept in therapeutics areas such as oncology, and infectious and rare diseases. As described above, LNP enabled the commercialization of the first siRNA drug, Onpattro [31]. Since then, a second siRNA product, Givlaari, has gained approval in the US for Acute Hepatic Porphyria [32]. Unlike Onpattro, this product does not use LNP for delivery, instead relying on conjugation of the siRNA to a triantennary GalNAc ligand which provides efficient uptake by the ASGPR receptors on hepatocytes. For hepatocyte delivery of siRNA, the field has gravitated towards the use of these conjugates as they enable subcutaneous administration (LNP require IV administration), steroid co-medication is not required, and they result in very long duration of action (Givlaari is dosed every month, but other conjugates such as Inclisiran – a PCSK9-targeting product in clinical development – have shown durations up to six months to enable twice yearly dosing [33,34]). These conjugates lack the active endosomal release mechanism of ionizable LNP, thus requiring higher dosages, but are generally safe with a wide therapeutic index. For these reasons we expect that GalNAc conjugate approaches will continue to dominate for delivery of siRNA and other small oligonucleotides for hepatocyte targets. One factor enabling the use of GalNAc with these NA is the ability to extensively chemically modify the NA molecule, using modified bases such as 2'-Ome and 2'-F. These modifications, among others, confer considerable stability to the molecules. Unlike siRNA or antisense, it is difficult to extensively modify MRNA (including replicons), (with the exception of Uridine modifications to abrogate immune stimulation) without impairment of translation [35]. Therefore, LNP are the delivery vehicle of choice for mRNA, and this is reflected in the number of mRNA-LNP products in development (Table 2). Due to their modular nature, LNP retain utility for the delivery of siRNA and oligonucleotides beyond the hepatocyte. LNP surface properties can be altered to change circulation times and, thus, tissue tropism to provide access to other cells such as the Hepatic Stellate Cells or tissues, such as various cancers, which is discussed in more detail below.

2.5. Formulation methods

An earlier review by MacLachlan in 2007 provided an excellent overview of historical methods of producing liposomes and lipid nanoparticles for nucleic acid delivery [36]. Essentially, the field was revolutionized in the early 2000's by the development of the spontaneous vesicle formation by ethanol dilution to produce "Stable Nucleic Acid Lipid Particles" (SNALP). Previously, the most common way to produce NA loaded lipid formulations was passive encapsulation of payload by hydration of a lipid "thin film" with buffer containing said NA. This produced a heterogeneous mix of lipid particles, which then had to be made homogeneous (in terms of size) by some form of physical particle sizing technique, commonly extrusion through polycarbonate filters. There were many issues with this method of preparation, the principal two being low encapsulation of nucleic acid (a very expensive material)

and difficulties in scaling up due to the specialized equipment and high pressures required. Numerous alternatives to this method were attempted using detergents to solubilize lipids [37,38] or by taking advantage of the solubility of lipids in ethanol, a water-miscible solvent, culminating in the above-mentioned Spontaneous Vesicle Formation by Ethanol Dilution technique.

The Ethanol Dilution technique is much more robust, reproducible and fully scalable, compared to the detergent dialysis technique. Briefly, this technique entails the dissolution of lipids in ethanol at the appropriate concentrations. This solution is then mixed under specific conditions (i.e., temperature, flow rate) with an aqueous solution containing Nucleic Acid at an acidic pH, using a T-tube mixer. As these two solutions are mixed, the ionizable lipid becomes positively charged and complexes with the negatively charged NA at the same time as the overall lipid solubility is reduced by the dilution of ethanol. This results in the formation of the nascent lipid-NA particles which are unstable and susceptible to particle size growth until a further dilution with neutral buffer is performed. This technique is applicable across a wide range of formulation conditions and types of NA payload, resulting in high NA encapsulation efficiencies (>80%). Important parameters to optimize through this process are the ionic strength and pH of the aqueous solution, as well as the lipidic content [36,39]. This technique is used for the production of Onpattro [9] and has been used for several other clinical-stage LNP programs. Recently, a microfluidic mixing technique has been developed as an efficient tool to make formulations quickly at small scale in the laboratory, and which provides LNP with similar properties to those made by larger scale apparatuses [40–42]. As Cullis and coworkers described in several morphological studies, LNP formulated either by the T-mixing or microfluidics-mixing techniques exhibit similar structures with an electron-dense core – contrary to the aqueous core observed in the case of liposomes – owing to the conformation of the ionizable lipid and its formed complexes with the NA payload [40,42,43]. The structure of the LNP core is dependent on the degree of saturation and charge of the ionizable lipid used. This core is surrounded by a monolayer of structural lipids (i.e., phospholipids, cholesterol) and surface-located PEG-lipids.

3. Hepatic delivery – mechanism of action

3.1. Cellular uptake

The first demonstration of hepatocyte-specific gene silencing through systemic administration of LNP was conducted in 2005, when Morrissey et al. reported a >1.0 $\log_{10}$ reduction in serum hepatitis B virus (HBV) DNA after three daily intravenous (i.v.) injections of 3 mg kg^{-1} in a mouse model of HBV replication [27]. The next year, Zimmermann et al. published the first proof of concept in non-human primates (NHP), presenting up to 90% silencing of apolipoprotein B (ApoB) mRNA by a single siRNA injection at a dose of 2.5 mg kg^{-1} [6]. Since then, several studies have established the potential of LNP for hepatic delivery of either siRNA or mRNA in different animal models, as well as in humans [13,18,44–51]. The mechanism behind the natural tendency of LNP to accumulate in the liver lies on the adsorption of serum proteins, predominantly apolipoproteins, on the surface of the LNP following their i.v. administration [52]. This "protein corona", as it is called, has been reported to be the determining factor for the nanoparticle's biological functions [53]. Specifically, it has been found that the LNP composition (i.e., the particle's surface charge, the amount and lipid chain length of the PEG-lipid, etc.) determines the enrichment of specific proteins on the nanoparticles' surface, with crucial effect on their cellular uptake and in vivo localization [53,54]. As mentioned above, the pH-dependent charge of the ionizable LNP allows them to travel as essentially neutral particles in the blood, where they interact mainly with apolipoprotein E (ApoE), facilitating their endocytosis via the low-density lipoprotein (LDL) receptor into the hepatocytes (Fig. 3). Akinc et al. were the first to describe this endogenous targeting mechanism
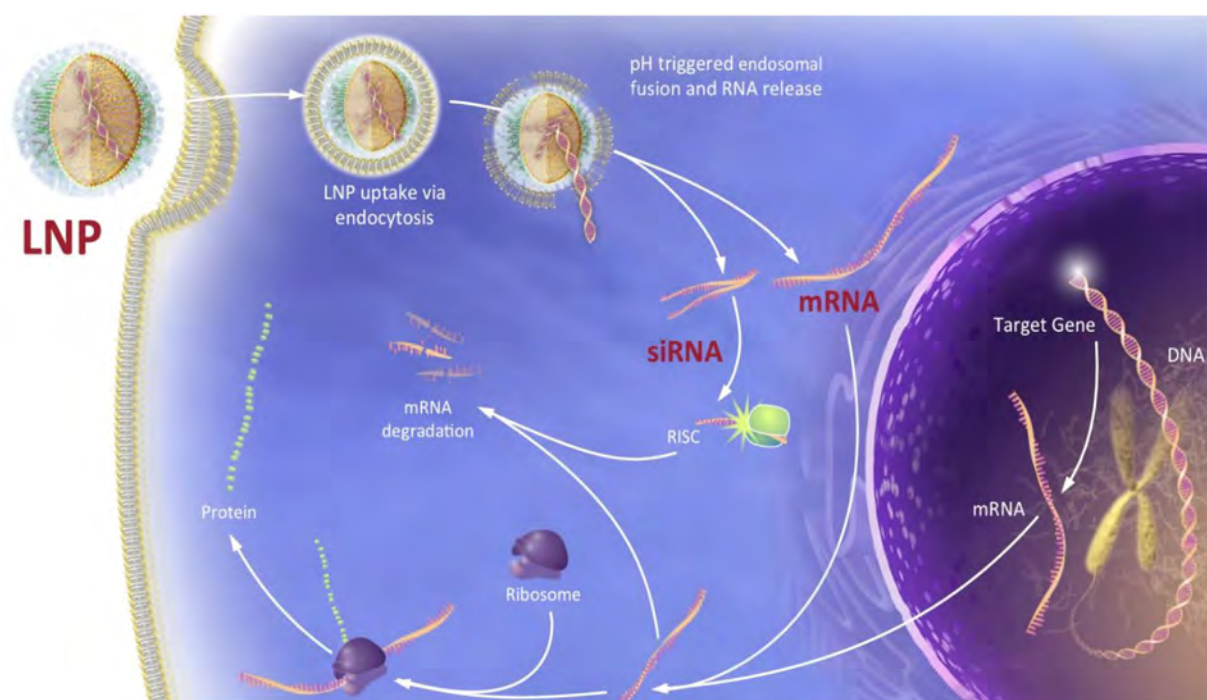
Fig. 3. Mechanism of action of the LNP formulation following systemic administration. LNP protect the RNA payload from nuclease activity and potentiate cellular uptake via endocytosis. Following the endocytic pathway, the pH of the maturing endosomes drops, triggering the LNP to fuse with the endosomal membrane and resulting in the release of the RNA payload into the cytoplasm where it is either incorporated into the RNA silencing machinery (siRNA) or is decoded in a ribosome to produce a specific polypeptide that later folds into an active protein (mRNA).

in 2010, after showing a 20-fold decrease in the cellular uptake of the i.v. injected LNP in ApoE-deficient mice, compared to wild type mice [55].

Later studies further suggested the involvement of distinct uptake mechanisms in the case of lipidoid-based LNP in comparison to the ionizable LNP [56,57]. The authors described the involvement of clathrin-mediated endocytosis (CME) and, subsequently, macropinocytosis in the cellular internalization of the ionizable (MC3 based) LNP, after reporting a 50-70% drop in LNP cellular uptake when they inhibited the respective mechanisms *in vitro* in HeLa cells [56]. Further testing the involvement of these mechanisms in the ionizable LNP activity showed that both mechanisms contribute to gene silencing, with macropinocytosis being the main mechanism [17,56]. On the other hand, the lipidoid-based LNP (i.e., C12-200-based LNP) employ only the macropinocytosis pathway to achieve cellular entry, followed by direct fusion with late endosomes, and subsequent escape to the cytosol or exocytosis of the unreleased siRNA load [57].

3.2. Endosomal escape

Following cellular internalization of the LNP, their success still depends on escaping the endosomal compartments to avoid enzymatic degradation and releasing their RNA cargo into the cytoplasm (Fig. 3). High-throughput confocal or electron microscopy techniques have allowed the visualization of LNP trafficking in live cells with traceable siRNAs, providing evidence of this cytosolic release during an early stage in endosomal maturation [56,58,59]. The main hypothesis for this mechanism is that the ionizable lipid component becomes protonated in the progressively acidic environment of the endosome, and associates with the negatively charged endosomal lipid bilayer. This leads to its destabilization through the formation of non-bilayer lipid structures [56,59,60]. As discussed above, this is governed by properties such as the ionizable lipid's pKa and structure of the hydrophobic domain [8] (Fig. 1B). However, only a small percentage of the total siRNA loaded into the internalized LNP is reported to actually reach the cytoplasm

through this mechanism [56,61,62]. Sahay et al. showed that 70% of the siRNA internalized into cells by LNP is exocytosed via a recycling pathway regulated by a lysosomal surface protein, called Niemann Pick type C1 (NPC1), known for its role in cholesterol trafficking. The rest of the endocytosed siRNA is subject to lysosomal degradation or other non-productive pathways [57]. In the case of the mRNA delivery, the percentage of mRNA released from endosomes appears to be higher than in the case of siRNA, with studies reporting up to 15%, depending on the fusogenicity of the ionizable lipid used [11,63].

In a different study, Patel et al. suggested that while siRNA requires endosomal escape at an early stage to achieve sufficient silencing, mRNA delivery and translation is favored by late endosome (LE) formation [61]. As a proof of that theory, a ~2-fold drop in protein expression was observed when mRNA-loaded LNP transfected LE-deficient versus wild-type cells. The authors pinpointed the issue to be the loss of mTORC1 (mechanistic target of rapamycin complex 1) signaling associated with late endosome formation, using mTORC1 inhibitors. Based on these results, they introduced the idea of co-formulating LNP with bioactive modulators of cellular signaling, like leukotriene inhibitors, resulting in a 2-fold enhancement of mRNA delivery in mice.

Maugeri et al. explored another hypothesis that part of the remaining RNA that wasn't released intracellularly was instead loaded into endosomal intra-luminal vesicles (ILVs), and released from the cells inside extracellular vesicles [64]. For this, human epithelial HTB-177 cells were transfected with human erythropoietin (hEPO) mRNA-LNP, comprising either DLin-MC3-DMA or DLinDMA. The transfected cells produced exosomes carrying hEPO mRNA, and ionizable lipids at a molar ratio of 1:1. The same was not observed when the exosomes were simply pre-incubated with the mRNA loaded LNP, implying a connection between the LNP endocytosis and the mRNA exocytosis. The authors further dosed the mRNA loaded exosomes intravenously in mice (at 1.5 µg of hEPO mRNA/mouse) and studied the protein expression in plasma and different organs, in comparison to mRNA loaded LNP. This confirmed that the mRNA loaded exosomes were able to deliver active

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mRNA, leading to hEPO protein production in different organs (mainly in the liver) – still 6-8 times lower than the level of protein produced by LNP – while inducing significantly lower inflammatory cytokine production that the LNP, owing to the lower amount of ionizable lipids present (1/3 of the respective amount in LNP).

3.3. Effect of particle size

Another important factor that drives the *in vivo* fate and may dramatically influence the potency of the LNP is their particle size. After the LNP are administered intravenously, they must pass through endothelial fenestrae of the liver to reach the hepatocytes. Owing to the limiting size of these fenestrations (100-110 nm in humans, and up to 160 nm in rodents), it is generally considered that LNP with sizes around 100 nm or less would have more chances of a successful RNA delivery [65]. As a first test of this hypothesis, Akinc et al. prepared siRNA loaded LNP with varying sizes (50-150 nm), using extrusion through different pore-sized membranes, while maintaining the exact lipid composition among the LNP, and dosed them systemically in mice at a dose of 3 mg kg^{-1} [66]. Upon doing so, the authors showed a clear improvement in hepatocyte gene silencing as the size decreased from 150 nm to 50 nm. Basha et al. came to the same conclusion when they tested the gene silencing capacity of LNP sized between 80-360 nm in hepatocytes and liver macrophages of mice. Delivery in the liver was favored when using smaller particles, while the opposite was true for macrophages [59]. In a later study, this conclusion was contradicted; LNP with sizes ranging from 27 to 117 nm were prepared by varying the content of the PEG lipid (0.25 to 5 mol% lipid) and evaluated for liver gene silencing activity following i.v. injection in mice [67]. All LNP showed similar accumulation to the liver, with the ~78 nm being the optimal size in terms of silencing potency. Still, both the smallest (27 nm) and the largest particles (117 nm) exhibited poor efficacy, owing to either stability issues (rapid dissociation of lipid components), or their inability to penetrate the dense vasculature of the murine liver, respectively. In a more recent study, the authors again varied the PEG lipid content (0.25-3%) of the LNP, resulting in hEPO mRNA loaded LNP with sizes ranging from 45 to 135 nm [63]. However, only minor differences in the cellular uptake where reported in this last study, depending on the size of the LNP, with the 65nm-LNP showing the highest hEPO expression in hepatocytes and adipocytes *in vitro*. These studies proved the importance of the particle's size in terms of its efficacy. However, as mentioned above, changing the mol% of the PEG lipid has a strong effect on the particle's *in vivo* fate, introducing an additional variable to the screen. In general, these conflicting results with respect to the optimal LNP size are an indication that multiple factors may contribute to an efficient LNP-mediated RNA delivery, such as LNP composition, PEG lipid content etc. Thus, further mechanistic studies are required to elucidate this size-activity relationship.

3.4. Limitations

3.4.1. Immunotoxicity

While the potency of a delivery system is important, safety is paramount, dictating the course of development, clinical translation and, ultimately, success. The identification of synthetic modifications for nucleic acid payloads has significantly reduced RNA-associated immune-stimulatory effects and considerably improved the immunogenic profile of LNP [68–71]. Despite this, administration of NA above a certain dose threshold in LNP format with most ionizable lipids has the potential to cause immune activation and cytotoxicity.

The innate immune system is activated when the phagocytic cells of the reticuloendothelial system (RES) recognize the lipid components of the LNP. Once activated, toll-like surface receptors (TLR2 and TLR4) can trigger the induction of high levels of cytokines, or cytokine release syndrome (CRS) [72,73]. Abrams et al. studied this phenomenon after the systemic administration of LNP composed of CLinDMA, seeing

production of both pro- and anti-inflammatory cytokines. These were primarily to the lipidic content of the LNP, and to a lesser degree, their payload [72]. In addition, the LNP may induce serum complement activation, leading to non-IgE-mediated hypersensitivity reactions, called complement activation related pseudoallergy (CARPA), and eventually to anaphylactic shock [74–76].

Efforts have been made to mitigate the inflammatory responses associated with LNP and increase their therapeutic window. These include pre-medication or co-administration with corticosteroids (e.g., dexamethasone) [45,72,77,78], use of pathway-specific inhibitors (i.e., Janus kinase (Jak) inhibitor) [77], as well as the employment of reinforced polyethylene glycol (PEG) shielding [75].

Immunosuppressive drugs like dexamethasone, a glucocorticoid receptor (GR) agonist, have been used to address immune stimulation in a large number of clinical and preclinical studies. Glucocorticoids affect the production of several pro-inflammatory factors, including cytokines, via the activation of the glucocorticoid receptor (GR) receptor in multiple cell types [79]. In 2010, Abrams et al. reported a significant reduction in the levels of at least six reported cytokines to near base levels, after treating mice with increasing doses (0.5 to 8 mg kg^{-1}) of dexamethasone intraperitoneally (i.p.) 1 hour prior to LNP dosing. No loss of gene silencing activity was observed [72]. Corticosteroid premedication has also been successfully applied in the clinic [45], however, it should be noted that chronic steroid medication can been associated with undesirable side-effects. A more recent work has shown that instead of pre-dosing the corticoid drugs, co-encapsulation and co-administration of a hydrophobic derivative of dexamethasone in the LNP allowed the suppression of the immune-stimulation at markedly lower dexamethasone dose (0.5 mg kg^{-1}), compared to the dose needed when the corticosteroid was administered alone (5 or 20 mg kg^{-1}) [78]. For this study, the authors prepared several hydrophobic derivatives of dexamethasone with single or multiple 18-carbon chains, with or without an ionizable amine, and studied their hydrophilicity/hydrophobicity, encapsulation efficiency, and biodegradability in LNP.

Tao et al. further showed that inhibiting the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, an important regulator of the immune system, can significantly improve the immunostimulant profile of the LNP [77,80]. The JAK-STAT pathway regulates the receptors for several cytokines that play a crucial role in several inflammatory responses. By pre-treating rats subcutaneously with 2 doses of a JAK inhibitor (6 hours apart) before the i.v. administration of the LNP (9 or 3 mg kg^{-1}), the authors showed 100% rescue of LNP-induced lethality, accompanied by a significant suppression of all associated toxic responses (i.e., cytokine induction, ALT and AST elevation etc.). This approach may have benefits over corticosteroids, which have multiple immuno-suppressive functions.

Shielding the particles with polyethylene glycol (PEG) has been another well established method to mediate these adverse effects and reduce the LNP interaction with serum proteins and complement [75]. Kumar et al. showed that increasing the PEG lipid mol% of the LNP from 1.5 to 10% reduced the production of cytokines, the complement activation and the recognition of the LNP from the macrophages in a murine *in vivo* model, but, as expected, also inhibited the LNP interaction with the ApoE in the blood, and, subsequently, their gene silencing efficacy. Importantly, in this study, the authors introduced the PEG lipid after the initial LNP formation to ensure similar sizes for the screened nanoparticles, while obtaining the desired PEG surface density [75]. These results showed that it is important to identify the PEG mol% that allows for maximum LNP protection from the immune system without sacrificing their activity.

3.4.2. Potential for immunogenicity

In addition to the potential for surface PEG to inhibit the cellular uptake of LNP and endosomal fusion, it has been widely reported that the PEG-lipid structure plays a strong role in determining circulation half life. Longer circulating particles can cause the production of anti-PEG

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immunoglobulin (Ig) M and G antibodies (anti-PEG IgM and IgG) from the B cells in the spleen, leading to a loss of potency upon repeated LNP administration, due to the Accelerated Blood Clearance (ABC) effect [29,81–84]. Dams et al. reported in 2000 a rapid blood clearance upon repeat dosing of empty PEGylated liposomes in rats and rhesus monkeys (but not mice), specifically when the second dose of liposomes was administered between 5-21 days after the first dose [81]. However, they claimed that nonantibody-soluble serum factors were responsible. Shortly thereafter, Semple et al. contradicted that conclusion by reporting for the first time the production of IgM antibodies from B cells after weekly dosing of PEGylated liposomes or lipid nanoparticles i.v. in mice [29]. Using different strains of naïve, T cell-deficient, or B and T cell-deficient mice, the authors showed that the rapid blood clearance was only seen in the naïve or T-cell deficient mice. It was not seen in the case of B and T-cell deficient animals, leading to the conclusion that the mechanism is B-cell and immunoglobin related. This was confirmed in the same study by Elisa-based IgM studies and supported by other publications around the same time [82,84]. IgM is a well known opsonin, capable of complement activation upon recognition of foreign bodies, leading eventually to phagocytosis [85]. Later that year, Judge et al. showed that these PEG-mediated antibody responses can be ameliorated by using "diffusible" PEG lipids with shorter dissociation times from the particle's surface, as discussed above [83]. To prove this hypothesis, the authors prepared a series of PEGylated liposomes containing PEG lipids with alkyl chains of different lengths (C14, C16 and C18) and reported a 10-fold reduction in the anti-PEG antibodies production at 7 days after their i.v. administration in mice, when a shorter alkyl chain (C14) PEG lipid was used versus a more stably integrated (C16 or C18) PEG lipid.

In a more recent study, the contribution of an alternative mechanism was proposed [86]. Results showed the effect to be driven initially by the activation of B-1 cells by the PC- containing LNP. This led to the production of anti-LNP (PC) IgMs and, eventually, to the production of anti-PEG IgM. Moreover, the authors demonstrated that by injecting the LNP with 2-week intervals or wash out periods between the injections, the ABC effect can be significantly reduced, owing possibly to the short biological half-life of the IgM. This last finding has been reported in previous studies as well, using PEGylated liposomes or polymeric nanoparticles instead of lipid nanoparticles [87,88]. It is important to note these studies were performed in animal models with no prior exposure to PEG molecules. In contrast, humans are increasingly exposed to numerous marketed products containing PEG, resulting in pre-existing PEG antibodies, which may present an extra hurdle when going to the clinic.

4. Going beyond the hepatocyte

4.1. Systemic delivery

The natural predisposition of LNP towards the liver has been shown to result in their accumulation in the hepatocytes, owing to mechanisms described above. However, structure-function based LNP design, or the use of active targeting ligands, can enhance delivery to a broad range of cells and/or tissues outside the liver. Apart from hepatocytes, which are the main type of hepatic cells, the liver microenvironment consists of other cell types, like Kupffer cells (liver macrophages), hepatic stellate cells (HSCs), endothelial cells among others. RNA delivery to these types of cells is of particular interest, owing to their involvement in several liver diseases. Delivery to these other cell types remains somewhat challenging, given the propensity for LNP to gravitate to the hepatocytes.

4.1.1. Hepatic stellate cells

There is considerable interest in hepatic stellate cells (HSC) owing to their strategic role in the regulation of liver damage and liver fibrosis [89]. Their physiologic function as liver pericytes lies mostly in the storage of vitamin A, the regulation of blood flow and turnover of

extracellular matrix (ECM) in the liver. However, upon liver injury they can become activated and differentiate into myofibroblasts, leading to the excessive production of ECM (mainly collagen type I) and profibrogenic cytokines, eventually causing liver fibrosis [89]. These cells are particularly hard to reach as they constitute only 5-8% of the resident cells in healthy liver, reaching around 15% in diseased liver [90]. To enable the targeting of these minority cells, several efforts have been reported by either employing active targeting molecules on the surface of LNP, or using rationally designed ionizable lipids that interact efficiently with HSCs among other hepatic cell types [91–95].

Sato et al. first attempted in 2008 to take advantage of the fact that HSCs are the main vitamin A storage site and used vitamin A (VitA) decorated liposomes carrying siRNA against HSP-47, a collagen chaperone glycoprotein, to target activated HSCs in both acute and chronic rat models of liver fibrosis [91]. Treatment with 5 or 6 repeated i.v. injections of VitA functionalized liposomes at a dose of 0.75 mg kg^{-1} led to localization of the liposomes in the liver, accompanied by significant fibrosis suppression in all three different fibrosis models employed in this study (induced by either dimethylnitrosamine (DMN) or carbon tetrachloride (CCl4) treatment or bile duct ligation (BDL)). These results revealed the potential of vitamin A to target both resting and activated HSCs. The mechanism behind this selective targeting was proven *in vitro* to be receptor mediated via the retinol binding receptor (RBR) in HSCs, although the contribution of vitamin A's hydrophobicity to nonspecific cellular uptake has not been completely excluded.

This targeting strategy was also employed in a recent study, where the authors employed VitA functionalized LNP comprised of hyperbranched lipoids to deliver a dual siRNA strategy (against type I collagen a-1 (Col1A1) and tissue inhibitor metalloproteinase-1 (TIMP1)) to HSCs in order to attack both the collagen production and promote collagen degradation in fibrotic mice (CCl4 treated) [96]. The targeted LNP showed clear superiority over the non targeted in terms of liver accumulation, which was more profound in fibrotic compared to naïve mice. This localization led to significant anti-fibrotic effects after repeat i.v. dosing at 1 mg kg^{-1} of each siRNA, based on the staining of fibrotic hepatic areas by Sirius Red. However, despite the high dose applied, no information was provided in regard to the silencing capacity of the delivered siRNAs *in vivo*.

In a different approach, authors attempted to incorporate VitA modified lipids (substituting the alkyl chain of the lipids with vitamin A) in their LNP and compared them to myristic acid and vitamin E containing LNP, in terms of silencing HSC specific fibrosis marker genes, like the Col1a1 in CCl4 treated mice [93]. As reported, at an i.v. dose of 0.5 mg kg^{-1}, only the VitA bearing LNP resulted in a reduction in COL1A1 levels in fibrotic mice. However, the decrease was moderate (~57%) and slow (72 h) possibly due to the long life of COL1A1 and the fact that vitamin A is incorporated in the LNP in this case and it is not available on the surface of the LNP.

As an alternative to Vitamin A, another study chose to explore a receptor that is overexpressed in activated HSC, the platelet-derived growth factor receptor β (PDGFR-β), using a cyclic oligopeptide, called pPB (C*SRNLIDC*) for the targeting of fibrotic HSCs [95]. The same approach had been previously reported to successfully carry i.v. dosed, interferon-γ loaded liposomes in the HSCs of thioacetamide treated fibrotic rats [97]. In this study, LNP carrying siRNA against HSP-46 were functionalized with the targeting peptide via a maleimide reaction and exhibited more than 2-fold higher accumulation in the liver compared to non-targeted LNP based on fluorescence tracking. Immunofluorescence staining analysis further revealed that the majority of the pPB-LNP could be found in the HSCs contrary to non targeted LNP.

However, it is critical to mention here that despite the benefits of selective protein silencing, with reduced off target effects, the use of exogenous targeting ligands introduces additional challenges with respect to manufacturing for use in the clinic. For that reason, studies have focused also on the use of ionizable lipids capable of interacting with HSCs without the need of exogenous HSC homing molecules. As an example,

C12-200 based LNP loaded with siRNAs against Col1a1 have exhibited significant localization and retention in the liver of fibrotic (CCl4 treated) versus nonfibrotic mice (i.v. dose of 3 mg kg^{-1}) with a specificity in non parenchymal cells and especially HSCs [92]. In a separate study, four weekly i.v. doses (0.1, 0.2 or 0.4 mg kg^{-1}) resulted in a significant, dose-dependent reduction in fibrosis (almost 50% decrease in collagen production) and Col1a1 mRNA knockdown (KD) up to 90% in CCl4 treated mice. Vollman et al., used C12-200 based LNP to identify novel targets in liver fibrosis [94]. By knocking-down genes that were upregulated during liver fibrosis and exploring the effect in collagen accumulation, the authors identified five genes that can act as therapeutic targets. From these, they reported for the first time that the early growth response 2 (Egr2), a protein with an essential role in peripheral nerve myelination and immune tolerance, had the most profound effect on fibrosis markers. According to their results, 94% knockdown of Egr2, using C12-200 based LNP (dosed i.v. on days 7 and 3 prior to CCl4 treatment, and at days 2, and 6 after CCl4 treatment at 0.5 mg/kg), resulted in an equivalent reduction in Col1a1 mRNA levels, and a reduction by half in collagen accumulation in CCl4 mice, compared to control animals.

4.1.2. Other hepatic cell types

Dalhman's group has done extensive research in trying to understand the LNP interaction with the liver microenvironment [98–103]. The group revolutionized the field by developing high-throughput screening methods through the use of unique DNA barcodes in LNP, allowing the parallel screen of hundreds of structurally-different LNP in terms of cellular biodistribution in a single experiment [100,104]. The DNA barcodes were 50-60 nucleotides long, including primer binding sites, random oligonucleotides and 10-nucleotide "barcode region" in the center of the sequence, and could be accurately identified at particularly low concentrations in isolated tissues or cell types, using deep sequencing. Moreover, loading the LNP with Cre recombinase (Cre) mRNA in combination with the barcoding technique enabled the evaluation of functional mRNA delivery to different cell types in Cre reporter mice [103]. This mouse model is genetically modified so that its cells fluoresce when Cre protein is expressed, allowing single cell resolution and increased sensitivity compared to other reporter mRNAs in regards to the location and the delivery of an active mRNA [105]. These experiments provided a wealth of information to help elucidate the relationship between LNP structure and cell-specific interactions.

Using this approach, the authors provided evidence that both MC3 and cKK-E12- based LNP interact with liver endothelial cells and macrophages in addition to hepatocytes [98]. Moreover, they were able to identify LNP formulations that delivered Cre mRNA preferentially to liver endothelial cells and Kupffer cells rather than hepatocytes, following the i.v. administration of 125 cKK-E12 based LNP containing cholesterol variants in mice [103]. From this comparison, they concluded that incorporating oxidized cholesterol, modified in its tail rather than at the sterol ring B, in LNP can alter the interaction of the LNP with serum proteins, affecting its biodistribution in preference to liver endothelial and Kupffer cells (4-fold higher Cre expression than in hepatocytes). The exact mechanism however behind this preferential distribution remains unclear. No relationship between LNP size and delivery was observed in this study.

In their latest study, however, they showed that the LNP biodistribution can change under conditions of mild innate immune activation, due to TRL4 activation caused by LPS pre-treatment in mice [101]. For this study the authors first employed cKK-E12 and MC3 based LNP, and showed a dramatic decrease in Cre protein expression in the hepatic cell types previously discussed (hepatocytes, endothelial cells, Kupffer cells) when the animals were pretreated with LPS causing TRL4 activation. Following a high-throughput screen, they identified a novel lipid, called cKK-E15 that exhibited specificity for liver macrophages. However, they observed the same drop in protein expression under inflammatory conditions, leading to the conclusion that the particles' biodistribution can be significantly altered under disease state.

4.1.3. Leukocytes

In a follow up study, the same authors revealed that it is possible for rationally designed LNP to deliver siRNA to T lymphocytes, without the need for targeting ligands [106]. Leukocytes including macrophages, monocytes, dendritic cells and lymphocytes are another major therapeutic target because of their central role in the immune system and their involvement in several diseases, such as autoimmune disorders, cancer, chronic inflammation etc. [107]. They can be found throughout the body, mostly in organs like the spleen or the liver, and also in the blood and lymphatic system. In this study, the authors screened 168 different LNP *in vivo* by varying i) the lipid head group (amines or boronic acid), ii) the lipid alkyl chain length, number and saturation (2 linoleic acid tails, 3 lipid tails, or an adamantane containing tail) iii) the phospholipid (DSPC versus DOPE), iv) the molar ratio of the components and v) the particle size (50-100 nm), in terms of their gene silencing capacity in T cells. Adamantane containing-LNP with DSPC showed the highest GFP silencing in murine splenic T cells (around 50% at a i.v. dose of 1.5 mg kg^{-1}), irrespective of their head group or size, followed by liver immune cells, and to a lesser extent splenic B cells. Interesting, no silencing was observed in hepatocytes. The authors concluded that a more "constrained" lipid tail, like adamantane, was essential for the LNP to target splenic T cells.

A previous study, however, had already discussed the possibility of actively targeting CD4$^+$ T lymphocytes, using surface functionalized LNP with anti-CD4 monoclonal antibodies (anti-CD4 mAbs) [108]. The antibody decorated LNP were capable of targeting CD4$^+$ T cells in different hematopoietic organs, not only in the spleen or blood circulation (i.e., lymph nodes and bone marrow). However, despite the impressive CD4$^+$ T cell affinity and selectivity, the level of silencing was limited in blood-circulating CD4$^+$ T cells, but significantly higher in the CD4$^+$ T cells isolated from the spleen of the treated animals.

The same group expanded the use of targeting mAbs towards inflammatory leukocytes, in order to deliver a therapeutic anti-inflammatory protein encoding mRNA, interleukin 10 (IL10) mRNA, against inflammatory bowel disease (IBD) in a mouse disease model [109]. For this, the authors employed a novel platform called Anchored Secondary scFv Enabling Targeting (ASSET) based on the incorporation of an antibody-binding lipoprotein in the LNP, allowing the non-covalent coating of the LNP with the targeting antibody [110]. As a result, the authors reported a significantly enhanced (20-fold) protein expression in the intestine and the spleen, accompanied by a 10-fold decrease of the protein expression in the liver. This enhanced protein expression in inflammation sites translated into reduced levels of proinflammatory cytokines and disease related symptoms.

Based on these encouraging results, Peer and colleagues decided to explore the combination of lipid design and active targeting using mAbs to enhance the LNP-mediated delivery of siRNA to leukocytes [111]. To do so, the authors designed a library of 14 structurally different ionizable lipids by varying the linker backbone, head group and alkyl chains of the lipids. Following their formulation characterization and *in vitro* evaluation, the authors proceeded to inject them systemically to mice in order to evaluate their biodistribution by tracking the fluorescently labelled siRNA in different tissues. By doing so, they concluded that lipids with piperazine head group localized preferably in the spleen (3-fold higher at 2 hours and 9-fold higher at 24 hours post i.v. administration, compared to liver localization) in contrast to lipids with tertiary amine head groups that were found mostly in the liver. Decorating the surface of the spleen-targeting LNP with an anti-integrin β7 mAb, resulted in significant CD45 mRNA knockdown (up to 50%) in both CD4+ and CD8+ T lymphocytes in the spleen and the lymph nodes, while no effect was observed for the isotype decorated LNP control. In the same study, they saw that using ethanolamine or hydroxylamine linkers was more efficacious in terms of gene silencing compared to hydrazine linkers. However, the silencing reported in this study remained limited, similar to previous studies using targeted LNP,

leading again to the conclusion that only a small percentage of the LNP are actually taken-up by these hard to transfect cells.

To avoid the possibility of increased clearance or immunogenicity associated with the use of full length antibodies, a different study employed a single chain antibody to target DLinDMA-based LNP to surface-expressed receptors of murine dendritic cells, such as the DEC205 receptor [112]. By doing so, the authors achieved up to an almost 70% knockdown of specific costimulatory targets (CD40, CD80, and CD86) individually or in combination, in splenic DC after the retro-orbital i.v. injection of the targeted LNP in LPS activated mice. This lowered the required dose compared to non targeted LNP. Using DEC205 deficient mice as a control further confirmed the receptor specific uptake. The authors showed that active targeting of DC could yield effective gene modulation of immune cells *in vivo*. Interestingly when the authors previously used full length DEC205 antibody as targeting molecule, they reported a significant loss of targeting potency in dendritic cells.

Collectively, these results show the potential for active or passive targeting to redirect LNP beyond the hepatocyte. While the doses required to mediate gene silencing in cells like leukocytes questions the readiness of these approaches to enter the clinic, the increasing research and understanding of the underlying mechanisms behind the biodistribution forecasts significant progress in the near future.

4.2. CNS delivery

Neurodegenerative disorders and brain cancer are among the most lethal and debilitating diseases worldwide, especially in senior populations. However, the development of effective therapeutics is significantly lacking in this field, mainly due to complexity of the brain, and the lack of drug delivery technologies capable of crossing the impermeable biological barriers associated to this area. The brain is tightly protected by the blood-brain barrier (BBB) that regulates the movement of proteins, ions, and information molecules between the systemic circulation and the central nervous system (CNS) with a unique selectivity, owing to its specific transporters and extensive tight junctions [113]. This makes the delivery of biomacromolecules from the blood to the brain particularly challenging. Further, i.v. administration of LNP favors their accumulation in the liver, resulting in low brain bioavailability. To circumvent these challenges, scientific interest has turned towards alternative administration routes that bypass the BBB, such as direct intracerebroventricular (ICV), intracortical (IC) or intrathecal (Ith) administration. The first attempt to deliver siRNA-LNP to the brain was reported in 2013 via IC or ICV injection [114]. For this application, the authors injected initially LNP loaded with siRNA against PTEN into the cortex of rats (IC dose of 2.5 µg of siRNA per animal in 0.5 µL dose volume). They reported the co-localization of LNP with neurons at a close distance from the injection point and ~90% silencing of PTEN in the area (<1 mm from injection point) that lasted up to 15 days. No immunotoxicity was observed. Knockdown of PTEN mRNA decreased further away from the injection site. In order to achieve a more spread distribution and knockdown, they proceeded to inject the LNP into the lateral ventricles (ICV injection) of the rat and managed to get ~50% knockdown of PTEN in different brain areas (striatum and dorsal hippocampus) at 4 times the distance from the injection site compared to IC injection. The therapeutic applicability of this administration route was further explored following the ICV injection of LNP loaded with siRNA against GRIN1 mRNA encoding for GluN1, an essential subunit for the N-methyl-D-aspartate (NMDA) receptors in the brain. NMDA receptors play an essential role in the synaptic plasticity and memory, but can be involved in several neuropathological conditions, thus their proper regulation is essential [115]. By doing so, the authors witnessed LNP localization 500 µm from the point of injection and almost 50% protein silencing 5 days post dosing. A later study used the same approach to deliver plasmid DNA (pDNA) encoding reporters like luciferase, or mCherry [116]. Significant LNP uptake was reported in astrocytes and

neurons when ionizable lipids containing vitamin E (versus vitamin A or myristic acid) were used, yet gene expression was reported only in astrocytes. More recently, the same group employed the same SS-cleavable lipid-based LNP to deliver eGFP or luciferase mRNA to the brain via ICV administration (1 µg of mRNA per mouse in 10 µL of volume dose) [117]. Through this study, the authors discovered that using a lower PEG density (1 versus 3 mol %) resulted in a higher activity. ICV administration of LNP bearing luciferase mRNA resulted in 7-fold higher activity than the lipofectamine complexed luciferase mRNA control, although it was accompanied by an elevation of IL-6 cytokine levels in brain lysates. Next, the authors used eGFP mRNA and showed that the expression of the protein could be found both in neurons and astrocytes. On the contrary, in their previous study the expression of mCherry from encapsulated pDNA was seen only in astrocytes, possibly owing to the inability of the LNP to reach the nucleus of neurons needed for pDNA protein expression.

The mechanism behind this preferential cellular localization in both astrocytes and neurons is believed to be ApoE-dependent [114,117]. The brain is rich in ApoE, which is produced mainly by astrocytes and secreted into the cerebrospinal fluid (CSF) [118]. In addition, astrocytes and neurons in the brain are known to express low-density lipoprotein receptors (LDLR) involved in the transportation of cholesterol [119]. The contribution of ApoE in the uptake of LNP by astrocytes and neurons was confirmed when decreased neuronal uptake of the LNP was observed *in vitro* in the absence of ApoE [114].

The potential to treat neurodegenerative diseases has also been explored following the intrathecal administration of the frataxin mRNA loaded LNP for Friedreich's ataxia (FRDA) [120]. FRDA is an inherited neurodegenerative movement disorder, caused mainly by the downregulation of a protein called frataxin (FXN). The authors achieved 3-fold higher FXN expression in the dorsal root ganglia of mice, a major pathological site in the case of Friedreich's ataxia, 24 h after they injected the LNP intrathecally into the lower lumbar region (L4-L5 or L5-L6) at a dose of 0.2 mg kg^{-1}, compared to the control group. Particles with lower PEG content (2.5%) were more active than smaller particles with higher PEG density (4%). Still no expression was observed in the spinal cord or the cerebellum. The therapeutic effect of this treatment was not shown in this study.

Despite the inevitable hepatic accumulation following i.v. administration of LNP, several studies have attempted to reach the CNS through the blood by targeting the receptors expressed on the BBB endothelium, without compromising the integrity of the BBB [121–123]. As an example, studies have employed oligopeptides, like angiopep, to surface-functionalize the i.v. dosed LNP towards the low-density lipoprotein receptor-related protein-1 expressed on both healthy brain endothelial cells and potential glioblastoma [121]. This promising targeting approach, however, was only validated *in vitro*. Around the same time, Conceição et al. proved for the first time that using active targeting can allow for LNP-mediated siRNA brain delivery upon i.v. administration *in vivo* [122]. In this integrated study, the authors used a rabies virus glycoprotein derived peptide (RVG) to facilitate the uptake of LNP by the BBB and deliver siRNA against the mutant ataxin-3 in two mouse models of Machado-Joseph disease (MJD). The edge of this approach was that RVG presents high specificity towards BBB, possibly through binding the nicotinic acetylcholine receptor, but can also facilitate neural cell entry after crossing the BBB [124]. The study indeed showed significant accumulation of the systemically administered LNP in the brain 3 hours post injection, which translated to around 32% silencing of mutant ataxin mRNA and protein aggregate levels in the cerebellum after 3 i.v. doses of 2.5 mg kg^{-1} of siRNA. That relatively moderate silencing resulted in more than 50% reduction of the pathological deficits of the disease in mice [122]. Still, a high amount of the LNP was found in the liver. A complementary study showed that the i.v. formulation was well tolerated since it did not induce the production of pro-inflammatory

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cytokines or microglia activation [125]. An increase in IL-6 was reported 4 hours after dosing, although no increase was observed upon repeated dosing.

In a more recent study, the authors chose to target receptors overexpressed in inflamed BBB, such as the vascular cell adhesion molecule-1 (VCAM-1), in order to deliver thrombomodulin (TM) mRNA encapsulated in i.v.-dosed LNP to the brain of a mouse model of acute brain inflammation [123]. Using LNP surface-conjugated with anti–VCAM-1 monoclonal antibodies resulted in 10-70–fold higher brain uptake (30 min post dosing of 8 μg of mRNA per animal) in healthy and intrastriatal TNFα-injured mice respectively, compared to IgG control LNP. This approach offered a very selective targeting along with a significant production of the TM protein, leading to ~ 84% alleviation of the brain vascular leakage. Taken together, these two studies highlight the importance of choosing targeting moieties with specificity towards receptors that are highly expressed in the BBB endothelium, instead of ubiquitously expressed throughout the body (i.e., Transferrin receptor (TfR), insulin receptor (IR), and LDLR) but also receptors that are available on the surface of the endothelial cells.

4.3. Lung delivery

The lung is also frequently targeted by nucleic acid therapeutic approaches, owing to both the many disease associated with it, and its seemingly accessible nature. However, the lung is a particularly well protected tissue, owing to the presence of complex biological mechanisms intended to remove exogenously inhaled particles, such as mucociliary clearance and alveolar macrophages engulfment. In 2018, Robinson et al. provided the first proof that LNP carrying cystic fibrosis transmembrane conductance regulator (CFTR) mRNA can circumvent these barriers and can be successfully applied for the treatment of cystic fibrosis following nasal administration in a mouse disease model [126]. Two consecutive doses of LNP at a dose of 0.1 mg kg^{-1} mRNA per day led to the restoration of chloride response in the airway epithelium of CFTR-knockout mice, that lasted up to 2 weeks post-dosing.

Alternatively, LNP could avoid the associated pulmonary barriers (i.e., mucosal barrier) and reach the lung endothelium through the blood by tuning the surface charge or through surface functionalization using antibodies, peptides, or small-molecule ligands [127,128]. An example of this active targeting approach was reported by the University of Pennsylvania (UPenn, USA) in collaboration with Acuitas therapeutics (British Columbia, Canada) who used mRNA-LNP coupled with monoclonal antibodies specific to the vascular cell adhesion molecule, PECAM-1 (platelet-endothelial cell adhesion molecule-1) in the lung endothelial cells [127]. By doing so, the authors achieved 200 times higher localization and 25 times higher luciferase expression in the lung with high specificity to endothelial cells, following the i.v. injection of mAb-LNPs compared to non-targeted LNPs in mice. This impressive lung localization was further proven to be independent of ApoE mediated mechanisms by reporting the same levels of lung targeting in ApoE-deficient mice. An alternative targeting strategy was presented recently by another group [128]. In this study, the authors described an approach called Selective Organ Targeting (SORT), according to which systemically dosed LNPs can be redirected to different organs like the spleen and the lung, by introducing permanently charged (cationic or anionic) lipids (termed SORTs) to the LNP formulation and modulating their molar percentage. Upon doing so, the authors showed an increasing accumulation and protein expression levels in the lung when increasing the molar percentage of permanently cationic lipid (DOTAP), without reporting significant toxicity. More importantly, this approach could be applied to different classes of LNPs, such as DLin-MC3-DMA- and C12-200- based LNPs.

4.4. Ocular delivery

Nanoparticle-mediated delivery of therapeutic biomacromolecules to the eye has accelerated over the last decade, with respect to treating both anterior and posterior segment ocular diseases. Recently the potential of LNP for nucleotide delivery was explored against posterior eye diseases following their subretinal injection [129,130]. This presents several challenges, such as anatomical constrains, the permeability and diffusion in the vitreous humor or the interaction and uptake by tight cellular membranes [131]. However, Patel et al. revealed in their study that LNP can overcome these barriers and effectively deliver mRNA subretinally in mice, with preferable protein expression in the retinal pigment epithelium (RPE) layer and the Müller glia [129]. For this study, the authors screened structurally-different ionizable or cationic lipids with respect to their distribution and protein expression in the mouse retina and concluded that ionizable lipids (i.e., MC3 and KC2) are the most potent in terms of protein expression 24 hours post-subretinal injection of 200 ng of luciferase mRNA per animal, possibly owing to their intrinsic endosomolytic properties. No signs of retinal toxicity were observed. To further explore their cellular localization, the authors employed mCherry mRNA LNP and proved a colocalization of the mCherry signal with the immunostained RPE and Müller glia. The mechanism behind this preferential localization was again postulated to be ApoE mediated; the Müller glia are responsible for the ApoE secretion in the retina and both RPE and Müller glia express LDL receptors. In a parallel study, Huang et al. studied the effect of the surface charge of the LNP on their retention and uptake by the retinal cells [130]. To maintain a constant particle size (~70 nm) while tuning their surface charge, the authors kept the same molar ratio of the cholesterol, PEG lipid and phospholipid contents, but varied the ratio of the cationic lipid (1,2-dioleoyl-3-trimethylammonium-propane, DOTAP) and the cholesteryl hemisuccinate (CHEMS) to obtain charges between -30 to +50 mV. By doing so, it was shown that positive LNP of around +35 mV resulted in higher retention time and retinal distribution and were able to deliver fluorescently labelled siRNA into the retina, highlighting the importance of the balance between enhanced retention and facilitated diffusion in the retina. However, the authors did not provide any proof of active siRNA delivery and its silencing capacity. These studies represent proof-of-concept showing the potential of LNP for treatment of ocular diseases, though further work remains to fully elucidate the underlying mechanisms.

4.5. Alternative delivery routes

Exploring alternative routes of administration for LNP technology will enable less invasive treatments, a vital aim for future research efforts. Oral delivery of NA, for example, could revolutionize how gastrointestinal (GIT) diseases are treated. Until recently it was unclear if LNP could be employed for oral RNA delivery and survive the harsh GIT environment, but now several nanoparticulate systems have been advanced towards that goal [132]. Whitehead and colleagues studied the stability and silencing capacity of siRNA-loaded LNP in vitro in the presence of simulated biological media containing proteolytic enzymes and mucin, the basic component of the intestinal mucus barrier [133]. They observed that LNP could not withstand these harsh conditions. When their in vivo fate was explored following their oral or rectally gavaged administration in fasted mice at 0.5 mg kg^{-1} of GAPDH siRNA, LNP uptake was observed by the intestinal epithelium, based on the signal of fluorescently labelled siRNA cargo, still no silencing was observed possibly due to the mechanisms studied in vitro (e.g., enzymatic hydrolysis, mucin entanglement, bile salts etc.). These discouraging results don't necessarily prohibit the use of LNP for oral delivery of nucleotides but highlight the need to protect the LNP against the harsh GIT conditions with either the use of pH-sensitive polymeric materials or capsules. This study is a good example of the importance of publishing negative data to help advance the scientific research.

In a previously published report, the authors introduced a step-by-step protocol approach for the use of LNP towards efficient myocardial delivery [134]. In this report, no results were disclosed, however the possibility of intramyocardial or intracoronary administration of a lipidoid-based LNP in rats was discussed. The importance of these alternative routes is that an intracoronary administration of LNP could allow for a non-invasive procedure in humans for the treatment of heart diseases, although much work remains to be done to enable it.

5. Pharmaceutical applications

The diverse biological roles of RNA have encouraged their use in a variety of therapeutic and preventative applications, many of which are already in the development pipeline or under clinical assessment [13,48–51,135,136]. Table 1 summarizes some of the recent pharmaceutical applications of RNA loaded LNP that have been reported in vivo.

5.1. mRNA vaccines – infectious diseases & cancer

Among these approaches, one particular field stands out; the use of mRNA for cancer immunotherapies and the generation of prophylactic vaccines against infectious diseases. mRNA vaccines are generally assigned into two distinct subcategories: the non-replicating and the self-amplifying mRNA (SAM) vaccines. Both encode antigen product (s), but SAM additionally encode viral RNA polymerase, effectively allowing to the payload to make copies of itself. This leads to enhanced duration, higher level of antigen expression and to a strong immune stimulation at a much lower dose than the non-replicating mRNA [137]. However, for the same reason SAM vaccines are much bigger in size. Further, the copies of the payload made in situ in the cell will not be chemically modified and together this leads to significantly higher immunogenicity than the non-replicating mRNA vaccines. This has to be taken into account when testing in patients, especially when multiple doses are required [138]. Both types of mRNA vaccines can be used to enhance immune stimulation in the case of infectious diseases, however only non-replicating mRNA vaccines are employed for cancer immunotherapies [139]. The cellular target for mRNA vaccines are immune cells, and specifically professional antigen presenting cells (APC) (i.e., dendritic cells, macrophages, B cells) due to their role in mediating immune responses [138].

mRNA vaccines are currently trending since they present distinct advantages over conventional or DNA-based vaccines. The concept is to deliver an mRNA encoding the desired antigen to the cytoplasm of the immune cells, in order to stimulate the immune system of the patient to attack the actual disease. mRNA vaccines do not need to reach the cell nucleus to induce protein expression, as DNA vaccines do, since they are translated in the cytoplasm. Further, they provide more specific, stronger and long-lasting immune stimulation. Safety is another valuable facet of mRNA vaccines; they are not infectious or in risk of genome integration and mutagenesis. In parallel, their in vivo half life is relatively short, allowing for more control in case of adverse effects. Finally, their manufacturing is rapid, cost-effective, reproducible, scalable and cell-free [139]. As an example of the flexibility and swiftness around the production of mRNA vaccines, Moderna, a Massachusetts-based biotechnology company, developed and manufactured a mRNA vaccine candidate (mRNA-1273) against COVID-19 during the 2020 pandemic just 42 days after the release of the virus sequence. It entered Phase I clinical trials just a few weeks later. Some reports claim that the inflammatory potential of lipid particles containing a nucleic acid payload can be beneficial for this specific application up to a certain point, but it can still be modulated with chemical modification [137]. These advantages highlight the immense potential of mRNA to revolutionize the field of immunotherapies with applications in cancer or infectious diseases.

As with most NA therapeutics, poor intracellular delivery and the risk of enzymatic degradation is a challenging, which LNP readily solve. The earliest reports of formulation-based mRNA vaccines date

back to 2000 from Curevac, a biopharmaceutical company from Germany that focuses on mRNA therapeutics [140,141]. Protamine was used to electrostatically complex and protect the mRNA cargo, leading to a specific immune response in vivo. Since then many more nanostructured systems have been tested for delivery of mRNA vaccines to the target cells (i.e., cationic liposomes, cationic nanoemulsions, cationic polymers, lipoplexes, etc.). Lipid nanoparticles gained increasing popularity over the last years [138,142,143]. In 2012, Novartis published a study describing the potential for a SAM-based LNP vaccine dosed i.m. (1μg) to elicit immune responses comparable to viral systems, without their risks and limitations [144]. A year later, they applied this mRNA vaccine technology encoding an influenza H1 hemagglutinin (HA) antigen from the H1N1 virus and reported the production of protective titers at 2 weeks after two immunizations (8 weeks interval) in mice, at a RNA dose of 1 μg (80-fold lower than the dose required when using non-replicating mRNA vaccine) [145]. The flexible manufacturing of mRNA vaccines allowed the production of this SAM vaccine in just 8 days following the sharing of the hemagglutinin (HA) and neuraminidase (NA) genes data from the avian influenza A (H7N9) outbreak in China. Following that study, Pardi et al. demonstrated robust immunity against Zika virus in non-human primates (NHPs) after intradermal (i.d.) vaccination of LNP encoding the pre-membrane and envelope glycoproteins of a Zika virus strain (50 μg mRNA per animal). This was the first study to prove efficient translation to NHPs [146]. The protective efficacy reported with the LNP system exceeded 50–100 times the effect seen when inactivated virus or DNA vaccines were used. The same year, Curevac showed that a single i.m. dose of non-modified, non-replicating mRNA loaded LNP induced the production of protective antibody titers against rabies and H1N1-HA in NHPs (separate single immunizations of 10 μg mRNA per animal) [147]. The LNP were well tolerated and allowed for multiple administrations. Immunity lasted up to 1 year and even exceeded the levels achieved with licensed conventional vaccines. Since then, Moderna has taken the lead in the development of LNP-based mRNA vaccines for infectious diseases with several studies both in clinical and preclinical stage over the past few years (i.e., against Zika virus, Influenza, Multiple Tick-Transmitted Flavivirus Infections, COVID-19 (Coronavirus) among others) [148–152]. Recently, the same company published an interesting study where 30 proprietary, biodegradable ionizable lipids were screened in terms of the LNP tolerability and immunogenicity after their i.m. administration in mice (2 doses with 3 weeks interval at 0.001 mg kg^{-1} mRNA encoding influenza HA genes) [153]. In this study, the authors noted a divergence in hierarchies for the i.v. and i.m. administered LNP compositions. They further noted that the compositions yielding the best expression did not necessarily yield the best immunogenicity [153]. They also showed that, contrary to some opinions, innate immune stimulation is not a prerequisite for immunogenicity, implying that the tolerability of the LNP can be improved without losing potency. According to this study, the pKa of the ionizable lipid appeared to be a determinant parameter for the behavior of the LNP following i.m. administration, with the optimal pKa range to be between 6.6 – 6.9 for immunogenicity (higher than the preferred pKa for i.v. dosing; 6.2 – 6.5). Size was also a crucial factor with the most potent LNP showing sizes between 75-95 nm, after testing LNP with sizes from 50 to 140 nm. The 5 lead LNP from this first screen were then screened for potency in NHPs following 2 i.m. doses of 5 μg modified mRNA encoding H10N8 influenza HA genes. However, in contrast to the murine data, no clear superiority was seen for these lipids in comparison to the benchmark, MC3.

Generally, most studies have concluded that lipid structure and LNP composition, particle size and surface characteristics greatly influence their efficacy as a vaccine. The route of administration is also important, with intramuscular (i.m.) and intradermal (i.d.) injection being used most frequently. To date, i.m. and i.d. routes have offered the highest level of immunity and longest duration of effect, in addition to being straightforward. The intradermal route of administration is also favorable owing to the high frequency of antigen-presenting cells in the

Table 1
Therapeutic and preventative applications of the lipid nanoparticle technology reported in literature in animal models.

Therapeutic modality	Indication	Target	LNP composition (ionizable lipid)	Targeting moiety	Administration route	Animal Model	Dose	Therapeutic outcome	Company/Academic institution	Reference
siRNA	Hepatitis B virus (HBV)	Two siRNA targeting HBV RNA	DSPC:Chol: PEG2000-C-DMA: DLinDMA (20:48:2:30 molar %)	-	i.v.	Mice	3 daily doses of 3 mg/kg/dose	>1.0 $\log_{10}$ reduction in serum HBV DNA	Protiva Biotherapeutics	[27]
	Hepatitis B virus (HBV)	Trio of different siRNAs targeting all four HBV transcripts	Proprietary LNP ARB-1740	-	i.v.	Mice	3 bi-weekly escalating doses (starting from 0.3, 1 and finally 3 mg/kg)	1.7-1.9 $\log_{10}$ reduction in serum HBV DNA and HBsAg	Arbutus Biopharma	[177]
	Hepatitis Delta Virus (HDV)	Trio of different siRNAs targeting all four HBV transcripts	Proprietary LNP ARB-1740	-	i.v.	Mice	3 bi-weekly doses of 3 mg/kg/dose	>2 $\log_{10}$ and >1 $\log_{10}$ reduction in HBV and HDV viremia respectively	Arbutus Biopharma	[178]
	Hepatitis B virus (HBV)	Trio of siRNAs against genotype A, B, and C viruses	YSK13-C3: Chol: PEG2000-DMG: PEG2000-DSG -triGalNAc (70:30:3:0.5 molar %)	Trivalent N-acetyl-D-galactosamine (GalNAc)	i.v.	Mice	Single dose of 5 mg/kg	>1 $\log_{10}$ reduction in serum HBV DNA and HBsAg	Hokkaido University	[198]
	Zaire Ebola virus (ZEBOV)	Polymerase (L) gene of ZEBOV	DSPC:Chol: PEG2000-DMA: DLinDMA (20:48:2:30 molar %)	-	i.v.	Guinea Pigs	6 daily doses 0.75 mg/kg/dose, starting 24 hours post infection	100% survival and complete protection from viremia	Protiva Biotherapeutics in collaboration with UTMB	[190]
	Zaire Ebola virus (ZEBOV, Kikwit)	Trio of siRNAs targeting the viral protein (VP) 24, VP35 and L polymerase protein	DSPC:Chol: PEG2000-DMA: DLinDMA (20:48:2:30 molar %)	-	i.v.	NHP	7 daily doses 2 mg/kg/dose, starting 30 min post infection	100% survival and complete protection from viremia	Tekmira Pharmaceuticals in collaboration with UTMB	[191]
	Zaire Ebola virus (ZEBOV, Makona)	Two siRNAs adapted to target the VP35 and L polymerase protein	Proprietary LNP TKM-130803	-	i.v.	NHP	7 daily doses of 0.5 mg/kg/dose starting 3 days post infection	100% survival and 4 log-reduction in plasma viremia	Tekmira Pharmaceuticals in collaboration with UTMB	[192]
	Sudan Ebola virus (SUDV)	VP35	Proprietary LNP TKM-130803	-	i.v.	NHP	7 daily doses of 0.5 mg/kg/dose starting 3 days post infection	100% survival and 4 log-reduction in plasma viremia	Arbutus Biopharma in collaboration with UTMB	[193]
	Marburg virus infection (MARV-Angola, MARV-Ci67 or MARV-Ravn	Two different siRNAs targeting the nucleoproteins (NP) NP-718 or NP-314	Proprietary LNP	-	i.v.	Guinea pigs	7 daily doses of 0.5 mg/kg/dose starting 1 hour post infection	100% postexposure protection MARV-Ci67 and MARV-Angola: NP-718 siRNA alone, MARV--Ravn: NP-718 and NP-314 siRNA	Tekmira Pharmaceuticals in collaboration with UTMB	[195]
	Marburg virus infection (MARV-Angola)	NP-718	Proprietary LNP	-	i.v.	NHP	7 daily doses of 1 mg/kg/dose starting 3 days post infection	100% postexposure protection and 4 log-reduction in plasma viremia	Tekmira Pharmaceuticals in collaboration with UTMB	[196]
	Marburg virus infection (MARV-Angola, or MARV-Ravn)	NP-718	Proprietary LNP	-	i.v.	NHP	7 daily doses of 0.5 mg/kg/dose starting 4-6 days post infection	100% survival (LNP treatment: MARV-Angola 4-days post infection and MARV-Ravn 6-days post infection)	Arbutus Biopharma in collaboration with UTMB	[197]

Table 1 (*continued*)

Therapeutic modality	Indication	Target	LNP composition (ionizable lipid)	Targeting moiety	Administration route	Animal Model	Dose	Therapeutic outcome	Company/Academic institution	Reference
	Hypercholesterolemia	Apolipoprotein (ApoB)	DSPC:Chol: PEG2000-C-DMA: DLinDMA (10:48:2:40 molar %)	-	i.v.	NHP	Single dose of 2.5 mg/kg	90% silencing of apolipoprotein B (ApoB) mRNA	Protiva Biotherapeutics in collaboration with Alnylam Pharmaceuticals	[6]
	Hypercholesterolemia	Proprotein convertase subtilisin/kexin type 9 (PCSK9)	Chol: mPEG2000-DMG:$98N_{12}$-5 (1)4HCl (48:10:42 molar %)	-	i.v.	Mice, Rats, NHP	Single dose of 5 mg/kg	50–70% reduction in PCSK9 mRNA levels. Up to a 60% reduction in plasma cholesterol concentrations	Alnylam Pharmaceuticals	[44]
	Cancer	PCTAIRE1 kinase	DSPC:Chol: PEG2000-DMG: proprietary lipid (7:33.5:1.5:58 molar %)	-	i.v.	Mice	Four injections (2 per week) at a dose of 0.5 mg/kg/dose	Significant decrease in tumor size and weight and a dramatic tumor apoptosis	Arcturus Therapeutics	[165]
	Leukemia	BCR-ABL oncogene	Proprietary LNP based on SUB9KITS™ lipid	-	i.v.	Mice	3 doses of 5 mg/kg/dose	Decrease in leukemia burden and 60% knockdown of the BCR-ABL oncogene	Hannover Medical School Precision Nanosystems University of British Columbia	[169]
	Hepatic fibrosis	Col1a1	DOPE:Chol: PEG2000-DMG:ssPalmX (X = Vit A) (30:40:3:30 molar %)	VitA	i.v.	Mice	0.5 mg/kg	Reduction ~60% in COL1A1 levels	Hokkaido University	[93]
	Hepatic fibrosis	Egr2	DSPC:Chol:C12-200: PEG2000-DMG (10:38.5:1.5:50 molar%)	-	i.v.	Mice	0.5 mg/kg on days 7 and 3 prior to CCl4 treatment, and at days 2, and 6 after CCl4 treatment	94% knockdown of Egr2, equivalent reduction in Col1a1 mRNA levels, and a reduction by half in collagen accumulation	Biogen, Inc. & David H. Koch Institute for Integrative Cancer Research	[94]
	Hepatic fibrosis	HSP-46	DSPC:Chol: pPB-PEG-DSPE: PEG2000-DMG:DlinMC3 (10:48:1:1:40 molar %)	pPB	i.v.	Mice	Dose of 0.023 mg/kg every other day for 2 weeks	2-fold increase in liver biodistribution and HSP-46 silencing compared to non-targeted LNP. ~40% KD of HSP-46 mRNA	East China Normal University	[95]
	Hepatic fibrosis	Two siRNAs against Col1A1 and TIMP1	C15-PA based LNP	VitA	i.v.	Mice	1 mg/kg per siRNA every other day for a total of 4 weeks	Significant anti-fibrotic effects based on the staining of fibrotic hepatic areas by Sirius Red	China Pharmaceutical University	[96]
	Machado-Joseph disease	Mutant ataxin	DSPC:Chol:C16- PEG2000-Ceramide: DODAP (22:45:8:25 molar %)	RVG	i.v.	Mice	3 doses of 2.5 mg/kg/dose	Accumulation in brain 3 hours post injection, 32% silencing of mutant ataxin mRNA and protein aggregate levels in the cerebellum	University of Coimbra, Portugal	[122]

mRNA	Respiratory syncytial virus	SAM encoding Respiratory Syncytial Virus Fusion Glycoprotien (RSV-F)	DSPC:Chol: PEG2000-DMG:DLinDMA (10:48:2:40 molar %)	-	i.m.	Mice	1 µg/animal	Immune responses comparable to viral systems, without the risks of the viral vectors	Novartis Vaccines and Diagnostics	[144]
	H7N9 influenza virus	SAM encoding influenza H1 hemagglutinin (HA) antigen from the H1N1 virus	DSPC:Chol: PEG2000-DMG:DLinDMA (10:48:2:40 molar %)	-	i.m.	Mice	Two immunizations (8 weeks interval) of 1 µg/animal	production of protective titers at 2 weeks at a RNA dose 80-fold lower than when using non-replicating mRNA vaccine	Novartis Vaccines and Diagnostics	[145]
	Toxoplasma gondii infection	SAM encoding NTPase-II antigen	DSPC:Chol: PEG2000-DMG:DLinDMA (10:48:2:40 molar %)	-	i.m.	Mice	Two immunizations (3 weeks interval) of 10 µg/animal	5-fold higher NTPase-II-specific IgG titers. Highest survival rate and significantly prolonged survival time than control group	Wenzhou Medical University, Wenzhou, China	[199]
	H10N8 and H7N9 Influenza Virus	SAM encoding hemagglutinin (HA) proteins of H10N8 or H7N9	DSPC:Chol: PEG-lipid: proprietary lipid (10:38.5:1.5:50 molar %)	-	i.m./i.d.	Mice, Ferrets, NHP	Mice: i.d. with 10, 2, or 4 µg/animal Ferrets: i.d. with 50 or 100 µg/animal NHP: IM or i.d. with 200 or 400 µg/animal	100% protection form virus, increased survival rate and Strong hemagglutination inhibition (HAI) Titers compared to control	Valera, A Moderna Venture	[148]
	Zika virus infection	ZIKV prM-E proteins	DSPC:Chol: PEG-lipid: proprietary lipid (10:38.5:1.5:50 molar %)	-	i.m.	Mice	One (prime) or two (prime-boost) doses of 2 or 10 µg/animal	Neutralizing antibodies (nAb) protected several mouse strains (100% survival). 50% neutralization titer of ~1/10,000. Effect even 14 weeks post dosing	Valera, A Moderna Venture	[149]
	Zika virus infection	ZIKV prM-E proteins	DSPC:Chol: PEG-lipid: proprietary lipid (10:38.5:1.5:50 molar %)	-	i.d.	Mice, NHP	Mice: 30 µg/animal NHP: 50 µg/animal	Higher nAb responses than viral or DNA vaccines Protection from virus for at least 5 months in mice and 5 weeks in NHP post vaccination	UPenn in collaboration with Acuitas Therapeutics and BioNTech	[200]
	Influenza virus	Full-length HA of H10N8	DSPC:Chol: PEG-lipid: proprietary lipid:GLA (9.83:38.5:1.5:50:0.17 molar%)	-	i.m./i.d.	NHP	50 µg/animal i.m./i.d. (additional boost at week 15) or 5 µg mRNA-LNP co-formulated with GLA per animal only i.m.	Robust germinal centers and B cell responses, including plasma cells seeding into the bone marrow	Moderna Therapeutics	[151]
	Influenza virus	H10 hemagglutinin (HA)	DSPC:Chol: PEG-lipid: proprietary lipid (10:38.5:1.5:50 molar%)	-	i.m./i.d.	NHP	50 µg/animal i.m./i.d. (additional boost at week 15)	Antibody titers > protective level for seasonal influenza transmission during the study period of 25 weeks. High protein expression in monocytes and MDCs	Moderna Therapeutics	[150]

(continued on next page)

Table 1 (*continued*)

Therapeutic modality	Indication	Target	LNP composition (ionizable lipid)	Targeting moiety	Administration route	Animal Model	Dose	Therapeutic outcome	Company/Academic institution	Reference
	Influenza virus	Full-length HA	DSPC:Chol: PEG-lipid: proprietary lipid (10:38.5:1.5:50 molar %)	-	i.m./i.d.	Mice, Rabbits, Ferrets	Mice: two i.d. doses (3, 10, or 30 μg) or i.m. (10, 30, or 90 μg) or single i.m. dose of 30 Rabbits: two i.d. at 50 μg/animal Ferrets: two i.d. at 60 μg/animal	High levels of nAbs in all species. Mice: Single immunization → Protection against H1N1 viral challenge at 5 weeks post immunization and two immunizations→ against H5N1 virus challenge at 4 weeks post immunization	UPenn in collaboration with Acuitas Therapeutics and BioNTech	[201]
	Human immunodeficiency virus type 1 (HIV 1)	Light and heavy chains of the broadly neutralizing anti-HIV-1 antibody VRC01	DSPC:Chol: PEG-lipid: proprietary lipid (10:38.5:1.5:50 molar %)	-	i.v.	Mice	1.4 mg/kg	~170 μg/mL VRC01 antibody concentrations in the plasma 24 h post injection, exceeding the IC50 of VRC01 Ab against a variety of HIV-1 isolates	UPenn in collaboration with Acuitas Therapeutics	[146]
	Chikungunya infection	Human antibody CHKV-24	DSPC:Chol: PEG-lipid: proprietary lipid (10:38.5:1.5:50 molar %)	-	i.v.	Mice, NHP	Mice: 2-10 mg/kg NHP: 0.5 mg/kg	High level of mAbs expression in both mice and NHP 100% protection against virus and arthritis and elimination of viremia in mice	Moderna Therapeutics	[152]
	Cancer	Ovalbumin (OVA) or tumor associated antigens TRP2 and gp100	DOPE:Chol: PEG-lipid: cKK-E12:SLS (26:40.5:2.5:15:16 molar %)	-	s.c.	Mice	10 μg/animal	Uptake in different immune cell populations (dendritic cells, macrophages, neutrophils, and B cells). Tumor shrinkage and prolonged survival benefit	David H. Koch Institute for Integrative Cancer Research	[157]
	Cancer	Caspase or PUMA	DSPC:Chol: PEG2000-DMG: DLin-MC3-DMA (10:38.5:1.5:50 molar %)	-	i.v.	Mice, NHP	Mice: 2 mg/kg NHP: 0.05mg/kg	Increased tumor targeting and apoptosis, while decreasing liver toxicity	Moderna Therapeutics	[161]
	Cancer	Anti-HER2 Antibody	cKK-E12 based LNP	-	i.v.	Mice	2 mg/kg	Production of antibody levels in the blood equivalent to the ones obtained with 4 times higher dose of the actual protein. Improved pharmacokinetics and longer duration of effect. 4 weekly doses LNP → significant suppression of tumor growth and increased survival	David H. Koch Institute for Integrative Cancer Research and Translate Bio	[162]
	Cancer	Ovalbumin (OVA)	DOPE:Chol: PEG-lipid: proprietary lipid: Pam3	-	i.m.	Mice	2 injections of 20 μg/animal (1-week interval)	Enhanced cellular immune responses and a staggering delay of tumor growth, compared to LNP alone	Ewha Womans University, Seoul	[159]
	Hemophilia B	Human Factor IX	DSPC:Chol: PEG-lipid: ATX (7:40:3:50 molar %)	-	i.v.	Mice	Single dose of 2 mg/kg or three repeat doses at 4 mg/kg (10-day intervals)	LNP were well-tolerated. 2-fold increase in protein expression compared to MC3-based LNP. Higher and longer therapeutic levels compared to the current standard of care. Repeated administrations led to consistent protein expression.	Arcturus Therapeutics	[47]

Methylmalonic Acidemia	Human methylmalonyl-CoA mutase (hMUT)	DSPC:Chol: PEG-lipid: proprietary lipid (10:38.5:1.5:50 molar %)	-	i.v.	Mice	Single dose at 0.5 mg/kg or 5 weekly doses of 0.2 mg/kg	75%–85% reduction in plasma methylmalonic acid, increased hMUT protein expression and activity in liver. Repeat dosing reduced circulating metabolites and dramatically improved survival and weight gain.	Moderna Therapeutics	[13,135]
Acute intermittent porphyria	Porphobilinogen deaminase (PBGD)	DSPC:Chol: PEG-lipid: heptadecan-9-yl 8-((2-hydroxyethyl) (8-(nonyloxy)-8-oxooctyl) amino)octanoate (10:38.5:1.5:50 molar %)	-	i.v.	Mice, Rabbits, NHP	Mice: 0.5, 0.2, 0.1, 0.05 mg/kg Rabbit: 0.5 mg/kg NHPs: 0.5 mg/kg	Dose dependent protein expression in liver. Protection against mitochondrial dysfunction, hypertension, pain and motor impairment	Moderna Therapeutics	[48]
Fabry disease	Human a-galactosidase protein	DOPE:Chol: PEG2000-DMG:C12-200	-	i.v.	Mice, NHP	Mice: 1.0 mg/kg NHPs: 0.1 mg/kg	~1,330-fold increase in serum GLA protein levels over normal physiological values	Translate Bio	[136]
Ornithine transcarbamylase (OTC) deficiency	OTC protein	Chol:PEG2000-DMPE-DOTAP:CHEMS (20:2.0:50:28 molar %) + GalNAc-targeted polymer	-	i.v.	Mice	3 mg/kg mRNA + 25 mg/kg polymer	4-fold higher OTC expression than control group (maintained up to 10 days post dosing) Repeat dosing for 12 weeks led to steadily high levels of protein expression	PhaseRx	[49]
Arginase deficiency	Arginase 1	Proprietary LNP	-	i.v.	Mice	Repeat dosing of 2 mg/kg up to 11 wk (3 days intervals)	100% survival, no signs of hyperammonemia or weight loss to beyond 11 wk, compared with control groups that succumbed to the disease on day 22. Restoration of urea cycle activity. No hepatotoxicity	Moderna Therapeutics	[51]
Classic Galactosemia	Human galactose-1 phosphate Uridylyl transferase (GALT)	DSPC:Chol: PEG-lipid: proprietary lipid (10:38.5:1.5:50 molar %)	-	i.v/i.p.	Mice	i.v.: 0.5 mg/kg Single or repeat doses (biweekly for 8 weeks) i.p (neonatal): 1 mg/kg	Single dose ➔ 84 % protein expression and activity compared to wild type. Repeated dosing ➔ >60% reduction in plasma and liver galactose (maintained through 4 doses) Increased survival rate after i.p. dosing in neonatal pups	Moderna Therapeutics	[50]
Cystic Fibrosis	CFTR	DSPC:Chol: PEG2000-DMG: DLin-MC3-DMA (10:38.5:1.5:50 molar %)	-	inhalation	Mice	Two daily doses at a dose of 0.1 mg/kg	Restoration 1/3 of normal chloride response in the airway epithelium, that lasted up to 2 weeks post-dosing	Oregon State University, Portland	[126]
Friedreich's ataxia	Frataxin (FXN) protein	DSPC:Chol: PEG2000-DMG: DLin-MC3-DMA (10:32.5:2.5:55 molar %)	-	i.th.	Mice	0.2 mg/kg	3-fold higher FXN expression in the dorsal root ganglia compared to the control group	Pfizer	[120]
Acute brain inflammation	thrombomodulin (TM) mRNA	LNP based on proprietary ionizable cationic lipid A-L01	anti–VCAM-1 monoclonal antibody	i.v.	Mice	8 μg/animal	70-fold higher brain uptake (30 min post dosing) compared to IgG control LNP. ~ 84% alleviation of the brain vascular leakage	Acuitas Therapeutics	[123]

Abbreviations: DSPC: 1,2-distearoyl-sn-glycero-3-phosphocholine, DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, Chol: cholesterol, PEG2000-DMA: 3-N-[(q-methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyrestyloxy-propylamine, PEG2000-DMG: 1,2-Dimyristoyl-sn-glycerol, methoxypolyethylene glycol, PEG2000-DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000], DLinDMA: 1,2-dilinoleyloxy-3-N,N-dimethylaminopropane, DLin-MC3-DMA: (6 Z, 9 Z, 28 Z, 31 Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino) butanoate,C15-PA: 15-carbon-chain derivative of polyamidoamine, NHP: non human primates, VitA: Vitamin A, i.v.: intravenous injection, i.m.: intramuscular injection, i.d.: intradermal injection, I.th: intrathecal injection, s.c.: subcutaneous injection, i.p.: intraperitoneal injection, HBsAg: surface antigen of the hepatitis B virus, Col1a1: Collagen, type I, alpha 1 gene, HSP-46: heat shock protein (HSP) 46,TIMP1: tissue inhibitor of metalloproteinases 1, Egr2: early growth response 2, RVG: rabies virus glycoprotein , VCAM-1: vascular cell adhesion molecule-1, SAM: self-amplifying mRNA vaccine, IC50: half maximal inhibitory concentration , GLA: glucopyranosyl lipid adjuvant, HA: Hemagglutination, MDCs: myeloid dendritic cells, CFTR: cystic fibrosis transmembrane conductance regulator, SLS: Sodium Lauryl Sulfate, gp100: glycoprotein 100, TRP2: tyrosinase-related proteins 2, HER2: humanized epidermal growth factor receptor 2, Pam3: tri-palmitoyl-S-glyceryl cysteine, ATX: Arcturus proprietary ionizable amino lipid, UTMB: University of Texas Medical Branch at Galveston, UPenn: University of Pennsylvania, Philadelphia.

skin, compared to the muscle [148,151,154]. Moderna scientists compared i.m. and i.d. routes for LNP dosing in mice at different dose levels and saw no significant differences in immunogenicity [148]. Similar protective titers were reported in NHPs even when different APC subsets were targeted [150,151]. Still in NHPs, i.d. delivery showed more rapid response [151]. Intravenous (i.v.) administration is less preferable, since it is associated with liver accumulation and is much less convenient. The systemic route is only selected when the goal is the slightly different approach of expressing an antibody in the liver [146,152]. In 2016, the University of Pennsylvania (UPenn, USA) in collaboration with Acuitas therapeutics (British Columbia, Canada), used this tactic to generate significant levels of an antibody against HIV-1, VRC01. They reported efficient immunity in mice against intravenous HIV-1 challenge, after the mRNA-LNP was dosed at 30 μg of mRNA per animal (approx. 1 mg kg^{-1}) [146]. Moderna scientists followed the same approach, using systemically dosed mRNA-LNP in order to achieve hepatic expression of a neutralizing human monoclonal antibody (CHKV-24) against the chikungunya virus (CHIKV), leading to high mAb expression levels in both mice and NHP, and showed complete protection against lethal disease and arthritis, and elimination of viremia in mice [152].

Apart from infectious diseases, the other most discussed research area for mRNA vaccines, with multiple clinical studies ongoing, is cancer. Owing to genetic and epigenetic changes, tumor cells upregulate expression of various proteins that are specific to this type of cells and can be used as tumor antigens. Taking advantage of this distinct behavior, mRNA cancer vaccines are designed to encode cancer-specific antigens in order to elicit a specific immune response by host T cells towards the tumor cells. Before mRNA vaccines, cancer treatment was dictated by the use of antibodies, cellular, viral or DNA vaccines and small anti-cancer molecules with limited success and long term side-effects [155]. More recently, several biotech companies have developed personalized mRNA cancer vaccines by sequencing the patients' tumor genome and using proprietary algorithms that can detect the relevant cancer mutations and protein markers. These can then be encoded by an mRNA payload that is delivered by LNP. The successful application of this approach in humans was recently described by BioNTech, a leading company in the field of cancer immunotherapies from Germany [156]. Although most of these approaches rely either on the direct local injection of naked mRNAs or the employment of ex vivo loaded DC precursor cells, several nanocarriers have been employed to optimize mRNA delivery and protection and already reached the clinic [NCT03323398, NCT03313778] [139]. Attempting to identify the optimal LNP composition for a high T cell response, Oberli et al. designed a library of ionizable lipids and developed LNP loaded with mRNA encoding for ovalbumin (OVA), by varying the components (i.e., phospholipid, cholesterol and PEG-lipid type) and the molar ratios used [157]. Interestingly, sodium lauryl sulfate (SLS) was used as additive, since it provided optimized particle characteristics. After the subcutaneous injection of 10 μg mRNA per mouse, the authors selected the cKK-E12 lipid from this screen, and they saw that decreasing its molar ratio from 35% to 10% led to a moderate increase of the mediated T cell response. The final LNP composed of DOPE:Chol:PEG2000-C14: cKK-E12:SLS at 26:40.5:2.5:15:16 molar percent and was formulated at a lipidoid to drug ratio of 10:1. The formulation was efficiently taken up by different immune cells *in vivo*, as proven by using mRNA coding for Cre-recombinase in Ai14D reporter mice. The therapeutic efficacy of this formulation was then proven in two mouse tumor models (i.e., transgenic OVA-expressing and aggressive B16F10 tumor model) using mRNA encoding different antigens depending on the model, resulting in tumor shrinkage and prolonged survival benefit.

As briefly touched on above, there are several ways to enhance the potency of the LNP, such as decorating the surface of the LNP with moieties that target surface-expressed receptors in immune cells, or co-administering the LNP with adjuvants, capable of enhancing the immune stimulation [138,158,159]. As an example, Verbeke et al. showed that co-delivering mRNA with a clinically approved TLR4 agonist, the

monophosphoryl lipid A (MPLA), inside DOTAP-based lipoplexes could induce innate immune activation without losing potency [158]. This approach has also been applied with LNP [157,159]. Oberli et al. incorporated the TLR4 agonist lipopolysaccharide (LPS) in LNP, by substituting 1% of the molar ratio of the PEG lipid. They showed slower tumor growth and longer survival in B16F10 tumor mouse model after six s.c. doses of 10 μg of mRNA per mouse, with 3-day intervals [157]. More recently, a TLR1 and TLR2 agonist, the lipopeptide tri-palmitoyl-S-glyceryl cysteine (Pam3C) SK4, was introduced in OVA mRNA loaded LNP. It led to enhanced cellular immune responses and a significant delay of tumor growth, compared to LNP alone, after two intramuscular injections of 20 μg mRNA per mouse with1 week interval [159].

Besides their contribution in mRNA cancer vaccines, LNP have allowed the non-toxic treatment and delivery of mRNAs encoding for cyto- or immuno-toxic proteins to the tumor site, while reducing off-target toxicity and side-effects. Cytokines can be used to activate immune cells to kill the tumor, but at the same time they can be extremely toxic when administered to the entire body. To tackle this issue, systemic administration of mRNA-LNP encoding Interleukin-12 (IL-12), a cytokine with known anti-cancer efficacy, resulted in the reduction of the liver tumor growth (after 3 weekly i.v. doses of 0.025 mg kg^{-1} mRNA) and the increase of the survival rate (after 9 weekly i.v. doses of 0.025 mg kg^{-1} mRNA) in a mouse model of refractory MYC-driven hepatocellular carcinoma (HCC). It seemingly avoided toxicity in healthy tissues for the first time [160]. Moderna scientists tried a different approach, developing an mRNA coding for a toxic protein (i.e., p53 upregulated modulator of apoptosis (PUMA)) with a strong pro-apoptotic role through mitochondrial dysfunction. To ensure that the protein would only be expressed in tumor cells in order to cause their self-destruction and not in healthy cells, they incorporated a microRNA (miRNA) strategy that enabled the recognition and degradation of the toxic mRNA in healthy cells [161]. The authors took advantage of the cell/disease-specificity of the endogenous miRNA expression and inserted perfectly complementary miRNA binding sites to the modified mRNA, in order to target miRNAs, like miR122, which is present only in healthy hepatocytes. The miRNA-mRNA combined sequences were loaded in LNP and administered intratumorally in mice. The insertion of the miRNAs sequences did not affect the mRNA efficacy, or the endogenous levels of the miRNAs. Endogenous miRNAs recognized the complementary sequences and suppressed the toxic mRNA translation in healthy hepatocytes, but not when taken up by tumor cells. This strategy can significantly increase the specificity of tumor targeted delivery and apoptosis, while decreasing liver toxicity. It can be also combined with multiple miRNA binding sites, broadening the number of tissues/cell-types that can be protected from the delivery of mRNA inducing cell self-destruction.

Following a different strategy, a recent study showed for the first time the potential of mRNA loaded LNP to express full-size antibodies with anti-cancer activity in the liver, like the humanized epidermal growth factor receptor 2 (anti-HER2) antibody (known as trastuzumab). This surpasses the challenges associated with therapeutic monoclonal antibody delivery [162]. Systemic injection of the mRNA-LNP at a dose of 2 mg kg^{-1} in mice resulted in the production of antibody levels in the blood equivalent to the ones obtained with 4 times higher dose of the protein, accompanied with improved pharmacokinetics and longer duration of effect. Furthermore, 4 weekly doses LNP at 2 mg kg^{-1} of mRNA resulted in significant suppression of tumor growth and increased survival of xenograft-bearing mice.

5.2. siRNA therapeutics – cancer

siRNA therapeutics have also been successfully applied in the battle against cancer by targeting cancer-causing gene mutations and the pathogenic mechanisms involved [163]. The potential of this approach has been proven by a number of siRNA-LNP-based treatments in completed clinical trials (Table 2). Alnylam and Tekmira jointly

Table 2
Clinical trials employing lipid nanoparticle technology for nucleotide delivery

Therapeutic modality	Name	Indication	Target	Administration route	Sponsor	Phase	Start Year	Status	NCT ID
siRNA	ALN-VSP02	Solid tumors	KSP/VEGF-A mRNA	Intravenous	Alnylam Pharmaceuticals	I	2009	Completed	NCT00882180
	PRO-040201	Hypercholesterolemia	ApoB	Intravenous	Arbutus Biopharma (as Tekmira Pharmaceuticals)	I	2009	Terminated	NCT00927459
	ALN-TTR01	Transthyretin (TTR) Amyloidosis	Transthyretin (TTR)	Intravenous	Alnylam Pharmaceuticals	I	2010	Completed	NCT01148953
	TKM-080301	Neuroendocrine Tumors (NET) and Adrenocortical Carcinoma (ACC)	PLK1	Intravenous	Arbutus Biopharma (as Tekmira Pharmaceuticals)	I/II	2010	Completed	NCT01262235
	ALN-PCS02	Hypercholesterolemia	PCSK9	Intravenous	Alnylam Pharmaceuticals	I	2011	Completed	NCT01437059
	ALN-TTR02	Transthyretin (TTR) Amyloidosis	Transthyretin (TTR)	Intravenous	Alnylam Pharmaceuticals	I	2012	Completed	NCT01559077
	TKM-100201	Ebola-virus infection	VP24, VP35, L-polymerase	Intravenous	Arbutus Biopharma (as Tekmira Pharmaceuticals)	I	2012	Terminated	NCT01518881
	ALN-TTR02	Transthyretin (TTR) Amyloidosis	Transthyretin (TTR)	Intravenous	Alnylam Pharmaceuticals	II	2012	Completed	NCT01617967
	Patisiran (ALN-TTR02)	TTR amyloidosis	TTR	Intravenous	Alnylam Pharmaceuticals	III	2013	Completed	NCT01960348
	TKM-080301 (TKM-PLK1)	Advanced Hepatocellular Carcinoma	PLK1	Intravenous	Arbutus Biopharma (as Tekmira Pharmaceuticals)	I/II	2014	Completed	NCT02191878
	TKM-100802	Ebola-virus infection	VP24, VP35, L-polymerase	Intravenous	Arbutus Biopharma (as Tekmira Pharmaceuticals)	I	2014	Terminated	NCT02041715
	ALN-TTR02	Transthyretin (TTR) Amyloidosis	Transthyretin (TTR)	Intravenous	Alnylam Pharmaceuticals	III	2015	Active, not recruiting	NCT02510261
	ARB-1467	Chronic Hepatitis B infection	HBV transcripts	Intravenous	Arbutus Biopharma	II	2015	Completed	NCT02631096
	TKM-130803	Ebola-virus infection	VP35, L-polymerase	Intravenous	Arbutus Biopharma (as Tekmira Pharmaceuticals)	II	2015	Completed	PACTR201501000997429
	DCR-PH1	Primary Hyperoxaluria Type 1 (PH1)	-	Intravenous	Dicerna pharmaceuticals	I	2016	Terminated	NCT02795325
	DCR-MYC	Hepatocellular Carcinoma	-	Intravenous	Dicerna pharmaceuticals	I/II	2016	Terminated	NCT02314052
	ARB-1740	Chronic Hepatitis B infection	HBV transcripts	Intravenous	Arbutus Biopharma	Ia/Ib	2017	Terminated	ACTRN12617000557336
	ND-L02-s0201	Idiopathic pulmonary fibrosis	HSP47	Intravenous	Bristol-Myers Squibb	II	2018	Active, recruiting	NCT03538301
	ALN-TTR02	Transthyretin (TTR) Amyloidosis	Transthyretin (TTR)	Intravenous	Alnylam Pharmaceuticals	III	2019	Active, not recruiting	NCT03862807
	ALN-TTR02	Transthyretin (TTR) Amyloidosis	Transthyretin (TTR)	Intravenous	Alnylam Pharmaceuticals	III	2019	Active, recruiting	NCT03997383
mRNA	VAL-506440	Influenza	H10N8 Antigen	Intramuscular	Moderna Therapeutics	I	2015	Completed	NCT03076385
	mRNA-1325	Zika virus	Zika virus antigenic proteins	Intramuscular	Moderna Therapeutics	I/II	2016	Completed	NCT03014089
	VAL-339851	Influenza	H7 antigen	Intramuscular	Moderna Therapeutics	I	2016	Active, not recruiting	NCT03345043
	mRNA-2416	Advanced/metastatic solid tumors or lymphoma	OX40L	Intratumoral	Moderna Therapeutics	I	2017	Recruiting	NCT03323398
	mRNA-1647 and mRNA-1443	Cytomegalovirus Infection	CMV proteins	Intramuscular	Moderna Therapeutics	I	2017	Active, not recruiting	NCT03382405
	VAL-181388	Chikungunya virus	Viral antigens	Intratumoral	Moderna Therapeutics	I	2017	Completed	NCT03325075
	mRNA-1653	Human Metapneumovirus and Human Parainfluenza Infection	Viral antigens	Intramuscular	Moderna Therapeutics	I	2017	Completed	NCT03392389
	mRNA-4157	Solid Tumors	personalized neoantigens	Intramuscular	Moderna Therapeutics/Merck	I	2017	Active, recruiting	NCT03313778
	MRT5005	Cystic Fibrosis	CFTR	Inhalation/nebulization	Translate Bio	I/II	2017/2018	Active, recruiting	NCT03375047

Table 2 (continued)

Therapeutic modality	Name	Indication	Target	Administration route	Sponsor	Phase	Start Year	Status	NCT ID
	MRT5201	Ornithine Transcarbamylase (OTC) Deficiency	OTC	Intravenous	Translate Bio	I/II	2018/2019	Withdrawn	NCT03767270
	mRNA-1944	Chikungunya virus	Anti-Chikungunya Virus Monoclonal Antibody	Intramuscular	Moderna Therapeutics	I	2019	Active, recruiting	NCT03829384
	mRNA-3704	isolated methylmalonic acidemia (MMA)	human MUT	Intravenous	Moderna Therapeutics	I/II	2019	Active	NCT03810690
	mRNA-3927	Propionic Acidemia	alpha and beta subunits of propionyl-CoA carboxylase (PCC)	Intravenous	Moderna Therapeutics	I/II	2020	Active	NCT04159103
	mRNA-1273	COVID-19 (Coronavirus) Infection	full-length, prefusion stabilized spike (S) protein	Intramuscular	Moderna Therapeutics	I	2020	Active, recruiting	NCT04283461

demonstrated the safety of siRNA loaded LNP targeting the vascular endothelial growth factor (VEGF), a regulator of tumor angiogenesis, and kinesin spindle protein (KSP), a metastasis mediator, in a Phase I study involving 40 patients that received doses as high as 1.5 mg kg^{-1}. Tekmira also tested LNP loaded with siRNA against PLK1 (Polo Like Kinase 1), a promoter of tumor cell proliferation, and showed good tolerance and antitumor activity in 44% of the enrolled patients with advanced solid tumors at doses up to 0.75 mg kg^{-1} [NCT01262235, NCT02191878] [164]. In a different study, the authors chose to target PCTAIRE1, a kinase that is overexpressed in several human cancers and its downregulation has been associated with apoptotic pathways [165]. Four i.v. injections (2 per week) of LNP loaded with siRNA against PCTAIRE1 at a dose of 0.5 mg kg^{-1} in tumor bearing mice led to a significant decrease in tumor size and weight and induced dramatic tumor apoptosis. Other approaches have included targeting ligands (e.g., transferrin, hyaluronic acid) and cell permeating peptides, like oleoyl-octaarginine (OA-R8) and protamine, to increase LNP potency [166–168]. Harashima's group used Random non-standard Peptides Integrated Discovery (RaPID) system to identify a non-standard macrocyclic peptide with great affinity for the epithelial cell adhesion molecule (EpCAM), a transmembrane glycoprotein that is overexpressed in different types of cancer, and showed a 100-fold increase in cellular uptake and an improved silencing efficacy by functionalizing PLK1 siRNA LNP surface with it (i.v. dose of 0.5 mg kg^{-1} siRNA) [167]. Cullis and colleagues recently reported that passively targeted LNP could be redirected to target human chronic myeloid leukemia (CML) cells *in vivo* [169]. The authors developed LNP comprised of a proprietary mix of lipids, including the SUB9KITS™ ionizable lipid, which is developed and supplied by Precision Nanosystems and loaded them with a leukemia-specific siRNA targeting a fusion oncogene. After administering 3 doses of 5 mg kg^{-1} i.v. in mouse xenograft model, they authors reported a decrease in leukemia burden and 60% knockdown of the BCR-ABL oncogene.

5.3. siRNA therapeutics – viral infections

Harnessing siRNA therapeutics to battle viral infections, like hepatitis B (HBV) and hemorrhagic fever viruses, has also been a promising therapeutic application of LNP. Treating these viruses is a global priority owing to their high lethality and/or transmissibility. HBV is a DNA virus of the hepadnavirus family and it can transmitted via infected blood, open sores, or body fluids [170]. It primarily attacks the liver and replicates in hepatocytes, causing chronic or acute disease [171]. According to World Health Organization (WHO), around 6% of the global population is living with chronic HBV and are at risk of serious liver infection, and, eventually, death caused by cirrhosis and hepatocellular carcinoma, the main outcomes of this disease [172]. siRNA can be used to target HBV DNA transcripts and interfere with HBV RNA production. However, this approach has to address multiple parameters involved in viral persistence and will likely require targeting more than one protein of the virus. The first demonstration that LNP-mediated siRNA delivery could be effective against HBV was published in 2005 by Protiva Biotherapeutics scientists in collaboration with Sirna therapeutics [27]. The authors encapsulated two HBV targeted siRNA molecules (i.e., HBV263 and HBV1583 siRNA) in DLinDMA-based LNP and reported a more than 1.0 log$_{10}$ reduction in serum hepatitis B virus (HBV) DNA after three daily i.v. injections of 3 mg kg^{-1} in a murine hydrodynamic injection (MHI) model of transient HBV replication, with no immune stimulation detected [27]. The therapeutic effect lasted up to 7 days after dosing and could be extended to 6 weeks post-dose following weekly dosing showing the viability of the approach [173]. A few years later, Protiva Biotherapeutics and Tekmira Pharmaceuticals merged, subsequently merging again to form Arbutus Biopharma, a HBV solutions company [174,175]. In 2015, Arbutus proceeded to clinical trials with ARB-1467, a LNP loaded with three siRNAs targeting all four HBV transcripts. In 2017, they reported up to 2.7 log reduction of

hepatitis B surface antigens (HBsAg) in chronic HBV patients with favorable safety profile [176]. Arbutus scientists also developed a second generation product with the same LNP composition but a different siRNA payload (ARB-1740) [177]. The siRNAs were designed based on 6000 publicly available HBV genome sequences, queried against human and NHP transcriptome and selected in order to broaden their therapeutic range against the diverse HBV variants present worldwide. This system led to a strong and dose dependent serum and liver HBV DNA and HBsAg inhibition (up to 3 $\log_{10}$) after a single i.v. dose (0.03-1 mg kg^{-1}) in a vector-based mouse model [177]. Repeat dosing of ARB-1740 was tested in a human chimeric mouse model of HBV, with 3 biweekly escalating doses (starting from 0.3 mg kg^{-1}, then 1 mg kg^{-1} and finally 3 mg kg^{-1}), leading to 1.7-1.9 $\log_{10}$ reduction in serum HBV DNA and HBsAg. This effect was enhanced when combined with the current standard-of-care treatment for HBV patients (i.e., a capsid inhibitor and pegylated interferon-alpha) and led to a more robust induction of innate immune responses in a human chimeric mouse model of HBV.

In a follow-up study, the same group demonstrated the applicability of the ARB-1740 agent against both HBV and hepatitis delta virus (HDV) viremia (2.3 $\log_{10}$ and 1.6 $\log_{10}$ reduction, respectively) when injected i. v. at 3 mg kg^{-1} per dose, for 3 bi-weekly doses, in dually-infected humanized mice (HBV and HDV) [178]. HDV is an incomplete virus that needs the HBV machinery to replicate and it is usually a co-infection or a super-infection in HBV carriers, leading to a higher risk of liver cancer and death compared to HBV mono-infection [179]. Having proven the potential of LNP in a highly unmet medical need, further efforts can be focused to study the combination of HBV- and HDV- targeting siRNA LNP or the synergistic use of LNP with agents such as pegylated interferon (IFN)-alpha, and nucleoside analogues.

The same company had previously applied its LNP technology against Ebola virus in collaboration with University of Texas Medical Branch [180]. Ebola (EBOV) and Marburg (MARV) virus are both members of the Filoviridae family [181]. Ebola has five known subtypes (i.e., Zaire, Sudan, Bundibugyo, Tai Forest and Reston) with several variants (e.g., Zaire Ebola Kikwit or Makona variants), while Marburg virus has one type with 2 lineages and several strains (i.e., Angola, Ci67, Musoke, Ozolins, and Popp or Ravn) [181–183]. Although distinct viruses, they present clinical similarities with symptoms like severe haemorrhagic manifestations, fever, body aches, neurological and gastrointestinal implications, which usually lead to death 8-9 days after symptom onset [184]. They can be transmitted through direct contact with infected biological fluids [185]. Both viruses have caused dramatic outbreaks in the past with mortality rates up to 90% [186]. Because of their high lethality and transmissible nature, they are at risk of being weaponized, and in fact this has already been documented for Ebola [187,188]. However, no effective vaccines and no approved post-exposure treatments exist today against filoviruses. According to Geisbert and coworkers, macrophages and dendritic cells appear to play a key role in virus replication and dissemination to the lymph nodes [189]. The virus replicates rapidly in different organs, with one of the early and primary sites being the liver [184,190].

In 2006 Geisbert's UTMB group, internationally recognized for its contribution to the filovirus battle, designed four siRNAs that targeted the replication machinery of the Zaire Ebola virus (ZEBOV), and specifically the polymerase (L) gene of ZEBOV. In collaboration with Protiva Biotherapeutics, they encapsulated them in cocktails or individually in DLinDMA-based LNP, and showed complete protection from viremia and 100% survival, when injecting the LNP in ZEBOV guinea pig (GP) model (0.75 mg kg^{-1} per dose, 6 daily i.v. doses), starting treatment at 24 hours post infection [190]. A few years later, the same collaboration demonstrated the first proof of siRNA-LNP potential in a more rigorous NHP model of the Zaire Ebola (Kikwit strain). Here, they used a pool of three siRNAs targeting the viral protein (VP) 24, VP35 and L polymerase protein, interfering with both the replication machinery of the virus and the ability of VPs to inhibit the host immune response [191]. 7 daily i.v. doses of 2 mg kg^{-1} siRNA starting 30 min after the virus challenge resulted in complete protection of the infected NHPs from death and base levels of viremia. Tekmira continued to optimize this formulation, improving the lipid composition with a more potent ionizable lipid and removing the VP24-targeting siRNA [180].

The group later adapted this LNP to target the Makona strain of Zaire Ebola, which was responsible for the 2013-2016 Ebola Outbreak in West Africa. This product, TKM-130803, demonstrated the versatility and rapid adaptability of the siRNA-LNP platform as mismatches in the original siRNA payload for the new strain of virus were quickly corrected. TKM-130803 was proven effective in NHP infected with EBOV Makona, conferring 100% survival against lethal challenge of the virus, even when dosed 3 days post infection [192]. Treatment with TKM-130803 (7 daily i.v. doses of 0.5 mg kg^{-1} of siRNA) resulted in 4 log-reductions in plasma viremia of the infected animals when compared to non treated animals that succumbed to the disease 8 days post infection. This study provided the first proof of the LNP therapeutic efficacy against this highly lethal disease in NHPs, but also the applicability of this technology against different strains of the virus. Other studies continued to support the utility of the platform; LNP loaded with VP35-siRNA alone were injected in NHPs infected with the Sudan species of Ebola virus (SUDV), following the same dosing schedule, again proved 100% effective [193].

Unfortunately human trials with TKM-130803 didn't show the same significant efficacy, quite possibly due to the lack of a proper study design during the dire virus outbreak [180,194]. In 2015, a single-arm phase II trial was conducted in Sierra Leone, where the TKM-130803 was administered i.v. at 0.3 mg kg^{-1} in 12 EBOV patients for 7 consecutive days [194]. Unfortunately, though, only 3 patients survived, a survival rate deemed insignificant from untreated patients. The LNP were well tolerated in all cases. It should also be noted that the patients chosen for the study already had extremely high viral loads, advanced illness (compared to other post-treatment therapeutics clinical trials) and irreversible organ damage. These circumstances possible concealed the potential of the LNP, and a more controlled and carefully designed study would be required to draw firm conclusions.

In parallel, the same group developed a siRNA-LNP against Marburg virus infection (MARV) and tested its potency in guinea pigs infected with a lethal dose of either MARV-Angola, MARV-Ci67 or MARV-Ravn virus [195]. 2 different siRNAs targeting the nucleoproteins (NP) that form the viral capsid (i.e., NP-718 or NP-314) were encapsulated either separately or in combination (1:1) in the LNP, in an attempt to provide broader-spectrum antiviral activity. The siRNAs were designed to target regions conserved across all major MARV strains. Interestingly, the LNP treatment (0.5 mg kg^{-1} per dose, 7 daily i.v. doses starting 1 h post infection) conferred 100% postexposure protection when the NP-718 siRNA was delivered alone in the case of MARV-Ci67 or the most pathogenic strain, MARV-Angola, whereas in the case of MARV-Ravn 100% survival was only obtained when the cocktail of the 2 siRNAs was used, despite one of them being less efficacious when used alone. This was the first time that complete protection against the most lethal strain of MARV (Angola) was reported in guinea pigs. Using the NP-718 siRNA–LNP, they also showed that results translated to the stringent NHP model of MARV-Angola infection, even when treatment began up to 3 days after infection [196]. The importance of this post-exposure timepoint (72 h) is that this is the earliest time point that the viral RNA can be detected in the blood, allowing diagnosis. Following the same experimental design (0.5 mg/kg of NP-718 siRNA per LNP dose, 7 daily i.v. doses), the authors reported 100% survival of rhesus monkeys infected with lethal doses MARV-Angola and MARV-Ravn, even when treatment began on days 4 and 6 post-infection, respectively [197]. This study highlighted the excellent potential of siRNA-LNP to confer complete protection against MARV-Ravn in NHPs even when dosed just 1 day prior to when the control animals began to succumb to disease. Together, the results summarized here strongly support the potential of siRNA-LNP as a post-exposure prophylaxis or anti-viral treatment, even when dosed later in the course of the disease.

6. From bench to bedside - current status of clinical trials

The concept of gene therapy was introduced in the 60s, while the RNAi mechanism was first uncovered in 1998, revolutionizing the gene regulation field. Six years later, siRNA therapeutics entered human clinical trials, following the injection of the naked siRNA directly into the vitreous of patients with wet age-related macular degeneration [NCT00722384]. Protiva Biotherapeutics developed the first LNP formulation for systemic delivery of siRNA to be used in the clinic. In collaboration with Alnylam Pharmaceuticals in 2009, they developed a product with two siRNAs against the kinesin spindle protein (KSP) and vascular endothelial growth factor (VEGF), for use in patients with advanced solid liver tumors [NCT 00882180] [164]. In 2010, Alnylam Pharmaceuticals initiated a Phase I clinical study that later demonstrated the potential of this technology for the treatment of polyneuropathy caused by transthyretin (TTR) amyloidosis [NCT01148953]. In parallel, Arbutus Biopharma applied its LNP technology against several diseases, focusing on cancer, rare diseases, hepatitis B and Ebola virus infections (Table 2). The company eventually decided to focus their efforts in targeting the Hepatitis B Virus (HBV), as discussed earlier. Following a Phase II study, they achieved significant reduction of HBV transcripts and antigens following single and multiple doses of a well-tolerated LNP formulation loaded with a trio of therapeutic siRNAs [NCT02631096].

Since then, the field has gained significant momentum with several companies and research institutions, such as Genevant Sciences, Moderna, Dicerna Pharmaceuticals, Translate Bio, Silence Therapeutics and Massachusetts Institute of Technology (MIT) among others, developing their own LNP technologies. As an indication of the aptitude of the LNP technology, LNP-mediated RNA therapeutics have been used in a rapidly expanding array of studies for diseases ranging from genetic disorders to cancer immunotherapies and viral infections over the last decade (Table 2). These scientific efforts culminated with the FDA approval of Patisiran, the first ever RNAi therapeutic based on lipid nanoparticle technology in 2018 by Alnylam Pharmaceuticals [31].

Learning from the struggles and difficulties encountered in lipid nanoparticle-mediated siRNA delivery and its clinical translation, mRNA therapeutics followed into human trials some years later when Moderna Therapeutics put LNP loaded with mRNA encoding influenza related antigens, intramuscularly, in healthy patients in 2015 [NCT03076385]. Following that study, Moderna Therapeutics designed and conducted several more human studies in the vaccine setting, focusing on viruses like Zika virus or Chikungunya virus (Table 2). It is considered a more feasible application of this technology owing to the lower amounts of delivered mRNA required to modulate the body's immunity against these diseases. Then in 2017, Translate Bio became the first company to clinically employ nebulized LNP (MRT5005) containing cystic fibrosis transmembrane conductance regulator (CFTR) mRNA for use in patients with cystic fibrosis [NCT03375047]. This work was based on previous research initiated by Shire Pharmaceuticals in collaboration with Ethris several years earlier.

7. Conclusion and future perspectives

The wide range of diseases that are being evaluated in clinical trials today reveals the potential that the LNP hold for RNA therapeutics. It is worth highlighting, however, that from over 30 RNA-based therapeutics in clinical trials over the last decade, only 2 RNAi products (i.e., Patisiran (Onpattro) and Givosiran from Alnylam) – and 1 LNP – have reached the market. This indicates the challenges inherent in the development of new technologies, and in particular the translation from animals to humans. With the initial recent approval of Onpattro, LNP delivery technology has been fully validated, and the technology is well positioned to enable the next wave of RNA therapeutics – including mRNA for vaccines, therapeutic protein production and gene editing applications. The foundational work described in this review will continue to be built upon, to further enhance the potency and tolerability of NA-LNP products and unlock applications outside the hepatocyte.

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JOINT APPENDIX 36

ARTICLE

On the Formation and Morphology of Lipid Nanoparticles Containing Ionizable Cationic Lipids and siRNA

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S Supporting Information

ABSTRACT: Lipid nanoparticles (LNPs) containing short interfering RNA (LNP-siRNA) and optimized ionizable cationic lipids are now clinically validated systems for silencing disease-causing genes in hepatocytes following intravenous administration. However, the mechanism of formation and certain structural features of LNP-siRNA remain obscure. These systems are formed from lipid mixtures (cationic lipid, distearoylphosphatidylcholine, cholesterol, and PEG-lipid) dissolved in ethanol that is rapidly mixed with siRNA in aqueous buffer at a pH (pH 4) where the ionizable lipid is positively charged. The resulting dispersion is then dialyzed against a normal saline buffer to remove residual ethanol and raise the pH to 7.4 (above the pK_a of the cationic lipid) to produce the finished LNP-siRNA systems. Here we provide cryogenic transmission electron microscopy (cryo-TEM) and X-ray evidence that the complexes formed between siRNA and ionizable lipid at pH 4 correspond to tightly packed bilayer structures with siRNA sandwiched between closely apposed monolayers. Further, it is shown that ionizable lipid not complexed to siRNA promotes formation of very small vesicular structures at pH 4 that coalesce to form larger LNP structures with amorphous electron dense cores at pH 7.4. A mechanism of formation of LNP-siRNA systems is proposed whereby siRNA is first sandwiched between closely apposed lipid monolayers at pH 4 and subsequently trapped in these structures as the pH is raised to 7.4, whereas ionizable lipid not interacting with siRNA moves from bilayer structure to adopt an amorphous oil phase located in the center of the LNP as the pH is raised. This model is discussed in terms of previous hypotheses and potential relevance to the design of LNP-siRNA systems.
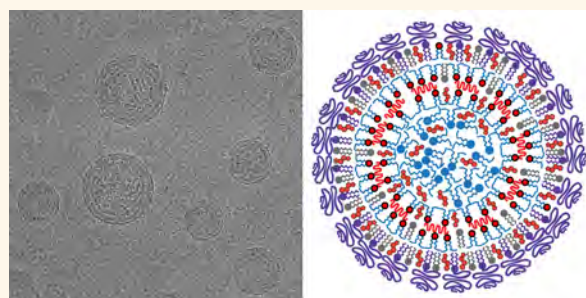
KEYWORDS: lipid nanoparticles, gene therapy, lipid biophysics, cryo-TEM, nanomedicine

In recent years the mantra surrounding gene therapy has been "delivery, delivery, delivery",[1−3] meaning that intracellular delivery of macromolecular RNA and DNA constructs into target cells was the primary impediment to practicing gene therapy *in vivo*. Viral vectors suffer from immunogenicity, manufacturing, and other concerns, whereas nonviral vectors have toxicity and potency issues. However, the recent successful Phase III trial of a lipid nanoparticle (LNP) formulation of siRNA to treat transthyretin (TTR)-induced amyloidosis suggests that nonviral vectors are starting to overcome the delivery barrier.[4] A key advance has been identification and incorporation of an optimized ionizable cationic lipid in the LNP-siRNA systems. Examples of such lipids are heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLin-MC3-DMA or MC3)

and 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA or KC2).[5,6] These lipids exhibit acid-dissociation constants (pK_a) below 7, ensuring a near neutral surface charge in the circulation upon intravenous administration, yet a strong positive charge at acidic pH to allow entrapment of nucleic acid polymers. Lipids such as MC3 and KC2 have been optimized for *in vivo* gene silencing in hepatocytes following intravenous (i.v.) administration and exhibit pK_a values in the range 6.2−6.7.[5]

Article
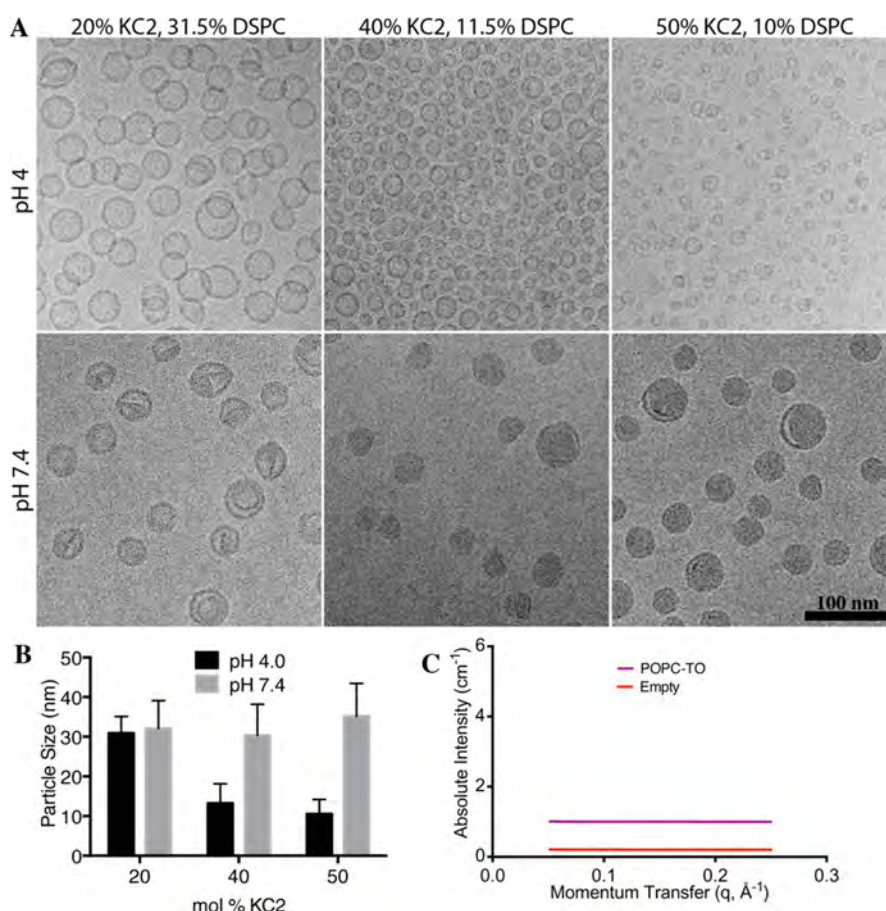
Figure 1. LNPs prepared in the absence of siRNA exhibit lamellar, vesicular structure at pH 4 and lamellar-solid core structures at pH 7.4, where the proportion of solid core increases as the KC2 content increases. LNPs composed of KC2, DSPC, Chol, PEG-lipid at a molar composition of 20−50/10−31.5/38.5−47.5/1.0−1.5, respectively, were formulated in the absence of siRNA. (A) Cryo-TEM was performed following dialysis to remove ethanol (pH 4), or dialysis against PBS to raise the pH and neutralize the KC2 (pH 7.4). Scale bar = 100 nm. (B) Particle sizing data for the respective formulations at pH 4 and pH 7.4. Results indicate mean ± s.d. (C) SAXS data for POPC/triolein/PEG-lipid LNPs (27/72/1 mol %) and "empty" LNPs composed of KC2/DSPC/cholesterol/PEG-lipid (50/10/38.5/1.5 mol %). In order to accommodate the SAXS data, each set was given an offset (Empty: 0.2, and POPC-TO: 1 units).

The physical processes by which these lipids enable entrapment and intracellular delivery of negatively charged polymers, and the structures formed, remain poorly characterized. LNP-siRNA systems are generated by rapid mixing of lipids in ethanol with siRNA in aqueous buffer (pH 4.0), followed by dialysis to remove ethanol and to raise the pH to 7.4.[7] The resulting structures display an electron-dense core as observed by cryo-TEM.[8] We have suggested that this core structure is generated through the initial association of ionizable cationic lipid with siRNA to form inverted micellar structures, followed by association with "empty" inverted micelles (formed from excess ionizable lipid) to form a hydrophobic core which is subsequently coated with more polar lipids (DSPC, PEG-lipids) as the polarity is increased.[2,7−10] However, this hypothesis does not account for certain features such as the fact that LNP-siRNA suspensions formed by rapid-mixing methods are initially transparent at pH 4 (indicating the presence of structures smaller than 30 nm diameter) and only become translucent upon dialysis, indicating the presence of larger structures. Moreover, alternative hypotheses of LNP structure[11] also do not necessarily consider these observations or reconcile all of the collected data.

Here, we re-examine the mechanism of LNP formation and the nature of the electron-dense structures formed using cryo-TEM and small-angle X-ray approaches. We show that LNP-siRNA

formed using ethanol dilution/rapid-mixing techniques displays a small multilamellar structure at high siRNA contents, where the nucleic acid is trapped between closely apposed lipid bilayers. At lower (clinically relevant) siRNA contents, LNP-siRNA systems exhibit a combination of siRNA-bilayer structure and an amorphous electron dense core, likely arising from an oil droplet consisting primarily of the neutral form of the ionizable cationic lipid.

RESULTS AND DISCUSSION

LNP Systems Containing KC2 Adopt Bilayer Structure at pH 4 and Amorphous "Solid Core" Structures at pH 7.4. Initial experiments focused on characterizing the morphology of LNP systems containing KC2 formulated in the absence of siRNA. When formulated using rapid mixing methods,[7,8,12,13] LNPs formed from lipid mixtures consisting of KC2, DSPC, cholesterol, and PEG-lipid (20−50/10−31.5/39−47.5/1.0−1.5 mol %) at pH 4 display bilayer vesicular structures (Figure 1A), where the size decreased as the KC2 content increased (Figure 1B). LNPs formed with 20 mol % KC2 exhibited a diameter of 30 ± 8 nm, whereas LNPs containing 50 mol % KC2 had a diameter of 11 ± 4 nm. When these formulations were dialyzed against phosphate-buffered saline (PBS) to bring the pH to 7.4, a progressive transformation to an electron-dense amorphous core

structure was observed as the KC2 content increased to 50 mol %. The observation that ionizable cationic lipids transform from small vesicular structures at pH 4.0 into much larger electron-dense core structures as the pH is neutralized suggests large-scale fusion of the small vesicles as the ionizable lipids adopt a neutral form. For the LNP systems containing 50 mol % KC2 (diameter 10.5 nm at pH 4, 35.1 nm at pH 7.4, see Figure 1B), assuming the cross-sectional surface area of a charged ionizable lipid is 0.7 nm^2, a lipid density of 0.9 g/mL, and a molecular weight of 590 g/mol, the "solid core" particles observed at pH 7.4 reflect fusion of some 36 vesicles observed at pH 4.

The solid core structure likely reflects an oil droplet phase formed from the neutral ionizable lipid as free-base KC2 adopts a liquid oil phase at room temperature. Previous work[14] using the rapid-mixing formulation process has shown that oil-in-water emulsions composed of POPC and triolein form "limit-size" solid core structures with similar morphology as the LNP systems containing 40 mol % KC2 at pH 7.4. The size of the POPC-triolein LNP increases as the core (triolein) to surface (POPC) lipid ratio is raised.[14,15] Similar behavior is observed for the KC2-containing LNP at pH 7.4. As shown in Supporting Figure 1, as the proportion of KC2 is raised from 40 mol % to 90 mol %, the LNPs (pH 7.4) increase in size from 30 to 90 nm, consistent with an increase in core lipid to surface (PEG-lipid, DSPC) lipid. Interestingly, small-angle X-ray scattering (SAXS) data of empty LNPs composed of KC2/DSPC/cholesterol/PEG-lipid (50/10/38.5/1.5 mol %) showed a similar scattering profile as POPC-triolein LNPs (Figure 1C), suggesting that no structured features are found within the nanoparticle.

Factors that could influence the structure of the amorphous core lipid structures observed at pH 7.4 include the unsaturation of the acyl chains of the ionizable cationic lipid and the charge on the cationic lipid. Increased unsaturation of the acyl chains has been shown to lead to higher levels of transfection[16] and increased propensity for formation of electron-dense core structure.[10] In order to determine whether reducing acyl chain unsaturation affected LNP structure, DODMA, which contains one unsaturated bond per acyl chain compared to two for KC2, was employed. LNPs were formulated with DODMA/DSPC/Chol/PEG-lipid over the range 20−40 mol % DODMA. As shown in Supporting Figure 2 broadly similar morphology was observed as for the KC2-containing systems, although it should be noted that DODMA has a higher apparent pK_a than KC2,[16] and thus at pH 7.4 is deprotonated to a lesser extent than KC2.

The lipid composition of the amorphous core structure is of interest. The dominant component is clearly KC2 as the proportion of the LNP adopting the core structure increases as the KC2 content increases (Figure 1A). The question is whether it is purely KC2 or whether other lipid components are present. Computer modeling places the PEG-lipid on the LNP surface[8] as does the direct influence of PEG-content on LNP size.[7,8,17] DSPC is likely preferentially located in the LNP surface monolayer as well given its amphipathic structure and is unlikely to be significantly soluble in a KC2 oil phase given the insolubility of diglycerides and triglycerides in bilayer membranes.[18] However, the solubility of cholesterol in a KC2 oil phase is not known and was therefore measured as indicated in Supporting Figure 3, leading to the finding that cholesterol has limited solubility in KC2. A solution of KC2 saturated with cholesterol at room temperature contains approximately 8 mol % of cholesterol. The amorphous core structure is therefore ascribed to the neutral form of KC2 containing a small amount of cholesterol.

LNP Systems Containing Permanently Cationic Lipids Do Not Exhibit Solid Core Structure. In order to demonstrate that the amorphous core is consistent with the presence of KC2 in the neutral form, the morphology of LNP formed when DOTMA (a permanently positively charged analogue of DODMA) was substituted for KC2, was investigated. As shown in Figure 2A, the behavior of LNP containing DOTMA at pH 4
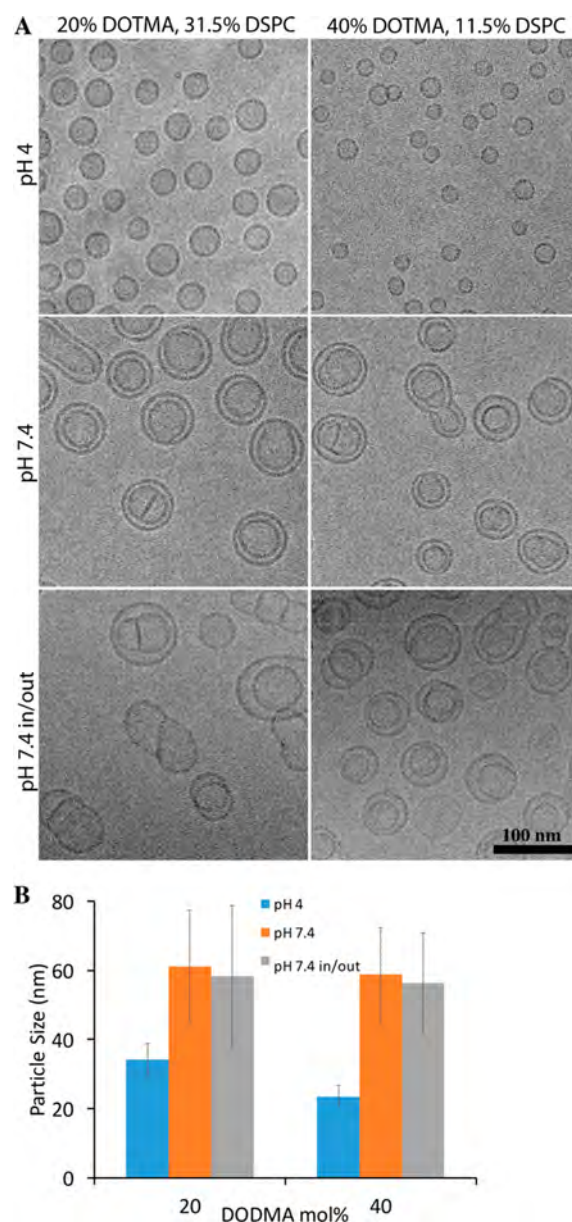
Figure 2. LNPs containing permanently positively charged lipid do not exhibit electron-dense core structure. LNPs composed of DOTMA, DSPC, Chol, PEG-DSPE at a molar composition of 20−40/11.5−31.5/47.5/1.0, respectively, were formulated in the absence of siRNA. (A) Cryo-TEM was performed following dialysis to remove solvent (while still at pH 4), or to neutralize the pH (PBS pH 7.4). Another set of formulations was generated in PBS pH 7.4 and dialyzed into PBS pH 7.4 to remove ethanol (labeled pH 7.4 in/out). Scale bar = 100 nm. (B) Particle sizing data for the respective formulations at pH 4 and pH 7.4. Results indicate mean ± s.d.

was similar to that observed for KC2-containing LNP, with smaller vesicular structures observed as the DOTMA content was raised from 20 to 40 mol %. The morphology observed on
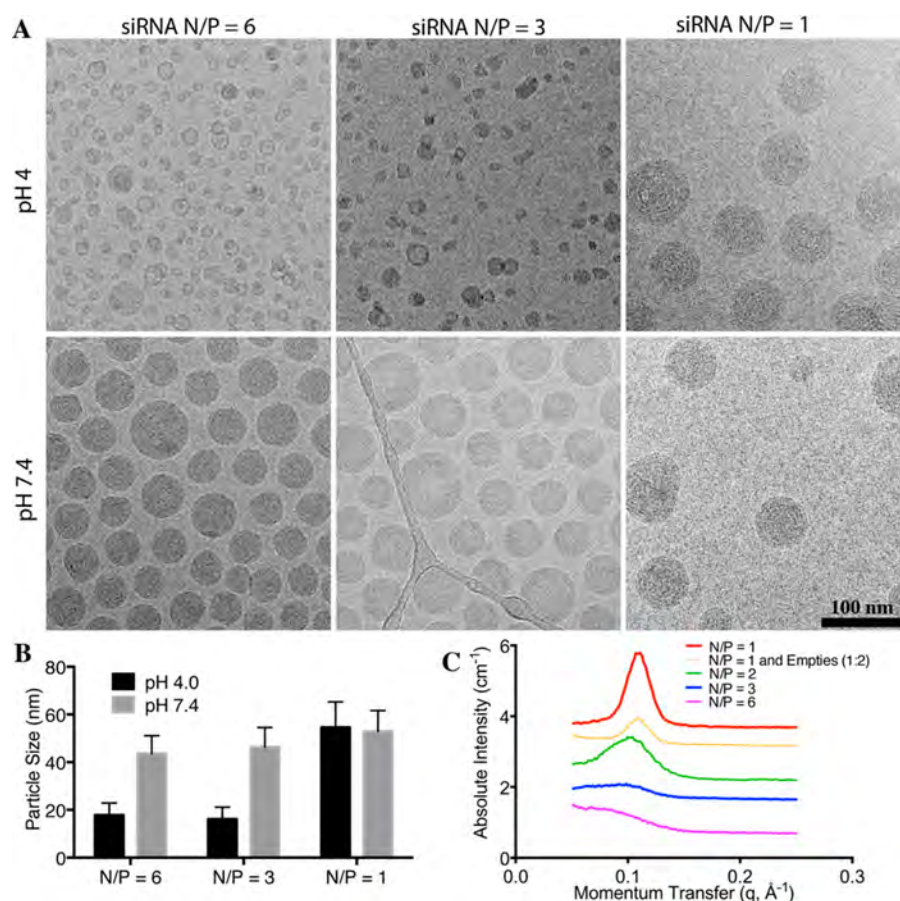
Figure 3. LNP prepared in the presence of siRNA exhibit stacked bilayer structure at high siRNA contents, where the proportion of stacked structures decreases as the siRNA content decreases. LNPs composed of KC2, DSPC, Chol, PEG-lipid at a molar composition of 50/10/38.5/1.5 were formulated at various charge ratios (N/P = 1, 3, or 6). (A) Cryo-TEM was performed following dialysis to remove ethanol (pH 4), or dialysis against PBS to raise the pH and neutralize the KC2. Scale bar = 100 nm. (B) Particle sizing data for the respective formulations at pH 4 and pH 7.4. Results indicate mean $\pm$ s.d. (C) Small-angle X-ray scattering data for LNP-siRNA formulations at pH 7.4. Formulations were generated at N/P = 1−6. A mixture of LNP-siRNA (N/P = 1) and empty LNPs was also analyzed. LNP-siRNA at N/P = 1 displays a scattering pattern characteristic of a bilayer structure closely supporting the cryo-TEM data. For the full scattering pattern ($q = 0.012−0.3$ Å^{-1}), refer to Supporting Figure 5.

dialysis against PBS pH 7.4 was, however, very different. In all cases a conversion from unilamellar vesicular structures to primarily bilamellar systems and an increase in particle size (Figure 2B) were observed, with no evidence for amorphous electron-dense core structures.

The results of this section lead to three conclusions. First, and most importantly, the amorphous "solid core" structure associated with LNP systems at pH 7.4 containing ionizable cationic lipids such as KC2 and DODMA at 20 mol % or higher is consistent with formation of oil droplets in the LNP interior consisting of the neutral (deprotonated) form of the ionizable lipid with a small proportion (8 mol %) of cholesterol. The second conclusion is that positively charged ionizable lipids, whether ionizable or permanently positively charged, adopt extremely small vesicular structures (diameter 15 nm or less) when dispersed from ethanol in aqueous buffer by rapid mixing. The reasons for the small size are not clear, but could be due to the relatively small headgroup of these lipids that leads to an inverted cone shape that is more readily accommodated in the inner monolayer of a membrane. A final finding concerns the conversion of unilamellar systems at pH 4 containing 20 mol % cationic lipid (either ionizable or permanently charged) to predominantly bilamellar systems when dialyzed against PBS.

This morphological change is clearly not due to the charge on the cationic lipid species, as it is observed for both ionizable and permanently positively charged lipids. In an attempt to determine whether the change in osmotic strength going from 25 mM at pH 4 to 160 mM in PBS could be driving the structural change, LNPs formed from DSPC/Cholesterol/PEG-lipid (55/44/1 mol %) were characterized. As shown in Supporting Figure 4a transition from unilamellar to bilamellar systems was observed for all cases where the exterior medium was of significantly higher osmolarity. The change in morphology appears to arise due to a fusion event and not deformation of vesicles due to osmotic effects, as the LNPs achieved following exposure to media of higher osmolarity are uniformly bigger than the initial structures.

LNP Systems Containing siRNA Contain a Proportion of Bilayer Structures. We next proceeded to characterize the influence of encapsulated siRNA on LNP structure. As shown in Figure 3A, at high levels of encapsulated siRNA (amino lipid nitrogen-to-siRNA phosphate (N/P) ratios of 1) where all of the positively charged ionizable lipid is complexed to an RNA phosphate, small multilamellar systems are observed. Such systems (albeit somewhat larger) have been reported previously for LNP containing ionizable cationic lipids and high levels of

antisense oligonucleotides, where the oligonucleotides reside at the interface between closely apposed bilayers.[19,20] It should be noted that in the present study, the formulations generated at N/P = 1 do not increase in size when the pH is neutralized (Figure 3B), whereas empty formulations or those at N/P = 3 or 6 increase in size when dialyzed into pH 7.4 buffer.

In an attempt to determine whether the LNP-associated siRNA remains complexed in bilayer structures at N/P values of 3 and 6 (where maximum *in vivo* gene silencing activity is realized),[7,17] SAXS studies were performed. As shown in Figure 3C for LNP-siRNA (pH 7.4) systems formulated at an N/P value of 1, scattering curves characteristic of the presence of closely apposed lipid bilayers with a repeat distance of 5.8 nm are observed. It should be noted that previous work[21] has shown that the complexes formed between cationic lipids and plasmid DNA generate lamellar structures with peaks in the range of $q = 0.1$ Å^{-1}. Comparatively, the presence of cubic phases in nanoparticles is observed at low q values (and visualized by cryo-TEM),[22] which is not observed here (Supporting Figure 5). This bilayer signature is also present for LNP-siRNA systems formulated at N/P values of 2, 3, and 6, albeit broadened with a decreased intensity. In order to show that the bilayer signatures at higher N/P values do not arise from a mixture of bilayer siRNA-containing LNP with empty LNP, the SAXS behavior of a mixture of LNP-siRNA systems (at N/P = 1) and empty LNPs at a ratio of 1:2 (w/w) was characterized. As shown in Figure 3C, while the resulting spectrum showed a decreased signal intensity relative to N/P = 1 formulations, there was no peak broadening.

As the siRNA content is reduced to N/P values of 3 and 6, the presence of lamellar structure induced by siRNA as detected by cryo-TEM is less definitive. In order to achieve improved resolution, LNP-siRNA systems were formulated and characterized for N/P values of 1.1 and 1.5, as shown in Figure 4
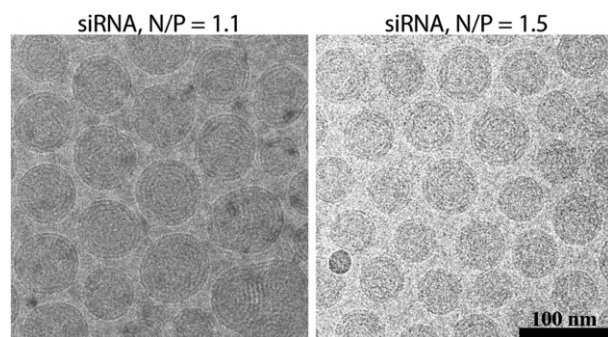
Figure 4. LNP-siRNA formulations generated at N/P values of 1.1 and 1.5 at pH 7.4 display stacked bilayer structures on the perimeter of the LNP. LNPs composed of KC2/DSPC/Chol/PEG-DMG (50/10/38.5/1.5 mol %) generated with siRNA at N/P = 1.1 and 1.5 were imaged by cryo-TEM. The resulting structures are multilamellar vesicles, with the number of resolved concentric rings decreasing at higher N/P ratios. Scale bar = 100 nm.

where bilayer structures are observable by cryo-TEM with progressively increased amounts of amorphous structure toward the center of the LNP. It is interesting to note that while siRNA induces bilayer structure (at pH 4) in LNP systems containing very high levels of KC2 and no bilayer forming lipid DSPC, such systems do not maintain bilayer structure at pH 7.4 and release all the associated siRNA as the pH is raised. This is shown in Supporting Figure 6 where, for LNPs composed of 98.5% KC2 and 1.5 mol % PEG-lipid, large stacked bilayer

structures are observed at pH 4, while at pH 7.4, amorphous electron-dense structures are seen with no entrapment of siRNA. This observation suggests a need for a certain amount of amphipathic, bilayer-forming lipid such as DSPC to provide the outer monolayer of the LNP-siRNA particle to stabilize the system and maintain internal siRNA-ionizable lipid stacked bilayer structure.

During the later stages of this study, an improved cryo-TEM instrument with higher acceleration voltages (300 kV) and better detection (direct electron detectors rather than CCD) became available. The higher acceleration voltage leads to decreased electron attenuation and improved sample penetration, thus improved imaging of the LNP core. It should be noted that the "solid-core" nature of lipid nanoparticles, as described in previous studies,[7,8,10,17] is influenced by the lower acceleration voltage (200 kV) and the amount of under-focus employed in those studies. Cryo-TEM imaging relies on defocus-enhanced contrast (*i.e.*, contrast is increased at the expense of resolution). Thus, the core of LNP-siRNA, as imaged by a 300 kV instrument, is observed as significantly less electron-dense than when imaged by a 200 kV instrument. The improved resolution possible with the 300 kV instrument clearly reveals that empty LNPs at pH 4 exhibit only bilayer structures, while those containing siRNA (N/P = 3) display lamellar phase within the electron-dense particles (Supporting Figure 7). At pH 7.4, however, the empty LNPs display surface bilayer structure with an amorphous core. Note that this surface bilayer morphology is not observed for LNPs that do not contain DSPC (Figure 5). LNP-siRNA systems with high siRNA contents (N/P ∼ 1) exhibit concentric bilayer ring structures consistent with the structures observed using the 200 kV instrument. Slightly higher N/P ratios (1.1−1.5) result in a combination of concentric ring structure and an amorphous core.

In summary, the results of this section indicate that at pH 7.4, LNP-siRNA systems formulated using the ethanol dilution rapid-mixing process consist of siRNA sandwiched between closely apposed bilayer structures preferentially located in outer layers of the LNP and that cationic lipid that is not associated with siRNA adopts amorphous solid core morphology in the center of the LNP-siRNA particle. These data also indicate that the DSPC-lipid preferentially resides on the surface of the LNP.

Implications for LNP-siRNA Structure and Design. The results of this investigation demonstrate that at high siRNA contents (N/P = 1), the LNP-siRNA systems formed at both pH 4 and 7.4 adopt a small multilamellar vesicle structure consisting of siRNA sandwiched between closely apposed concentric lipid bilayers. Conversely, LNPs formed in the absence of siRNA at pH 7.4 exhibit an amorphous hydrophobic lipid core consistent with an oil-in-water dispersion. At N/P values of 3 and 6 (which correspond to formulations used clinically) where there is an excess of ionizable cationic lipid, siRNA remains sandwiched within bilayer lipid assemblies (as indicated by small-angle X-ray studies), whereas the LNP core displays amorphous structure consistent with the presence of oil-phase lipid. For systems where there is only a slight excess of ionizable cationic lipid (N/P of 1.5), outer regions of the LNP display concentric ring structure, whereas the LNP center displays amorphous structure. On the basis of these observations we propose a revised model of LNP-siRNA structure for therapeutically active formulations, as shown in Figure 6, where the bulk of the ionizable cationic lipid segregates into a central oil phase and stacked bilayers of lipid-siRNA aggregates are located toward the periphery of the LNP.
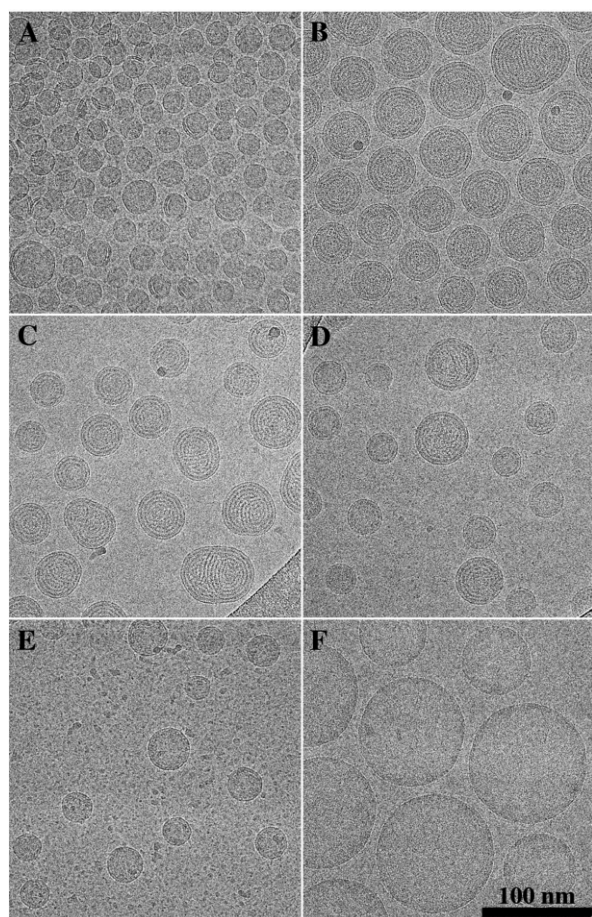
Figure 5. Cryo-TEM imaging with improved resolution supports the presence of DSPC-lipid on the surface of the LNP. All formulations, at pH 7.4, were vitrified and subjected to cyro-TEM using a 300 kV instrument with direct electron detectors. (A) LNPs composed of KC2/DSPC/cholesterol/PEG-DMG (50/10/38.5/1.5 mol %) generated without siRNA. LNPs of a similar composition were also formulated with siRNA at N/P = 1 (B), 1.1 (C), 1.5 (D), and 6 (E). (F) LNPs composed of KC2/PEG-DMG (98.5/1.5 mol %) were generated without siRNA. Scale bar = 100 nm.

The structure presented in Figure 6 differs significantly from the structure proposed previously indicating a nanostructured core of LNP-siRNA systems, where siRNA is encapsulated in inverted micelles in the LNP interior in a "currant bun" configuration and excess ionizable cationic lipid that is not complexed to siRNA displays inverted micellar structure.[7,8] This structure was suggested largely by molecular-modeling approaches. All other experimental data presented by Leung *et al.*[8,10] is fully consistent with the model presented here.

The revised structure suggests a number of ways to optimize LNP stability and possibly performance. First, the proportions of DSPC, cholesterol, and ionizable cationic lipids in LNP-siRNA systems have previously been developed through phenomenological approaches to optimize FVII gene silencing potency *in vivo*. The results presented here suggest that optimized ratios derived from these studies should be refined on the basis of the solubility of the lipid components with one another. For example, the optimized ratios for maximum gene silencing potency in hepatocytes in a mouse model are ionizable cationic lipid/DSPC/cholesterol/PEG-lipid (50/10/38.5/1.5 mol %).[5] However, if cholesterol is only 8 mol % soluble in the ionizable lipid oil phase and present at equimolar levels in the DSPC surface

monolayer, only 14 mol % of the total cholesterol is accounted for. The remaining 24.5 mol % cholesterol could potentially form crystalline structures and introduce particle instability. Similarly, if DSPC is primarily located in the outer monolayer in equimolar concentrations with cholesterol, an equilibrium size for an LNP containing 10 mol % DSPC would be ~80 nm diameter. Previous work has shown that LNP-siRNA systems containing 10 mol % DSPC exhibit maximum activity for a size of 80 nm diameter.[17] This suggests that to obtain smaller systems with optimized activity, higher levels of DSPC should be incorporated.

It should be noted that while the size of LNP-siRNA systems is controlled by the PEG-lipid content,[7,23] LNP systems that do not contain sufficient DSPC to cover an external surface monolayer will, of necessity, incorporate additional cholesterol and/or ionizable lipid in that monolayer. The PEG-lipid content determines the size of the LNP by virtue of its ability to inhibit further LNP fusion at some critical concentration as the small particles generated at pH 4 coalesce to form the larger LNP observed at pH 7.4. If the particle does not contain sufficient amphipathic lipid to cover the outer surface, it will exist in a metastable state stabilized by the PEG-lipid coat. In the absence of PEG-lipid, further rounds of fusion would be expected to occur until the exterior monolayer contains sufficient amphipathic lipid that additional fusion is inhibited. When the diffusible PEG-lipid dissociates from the LNP following i.v. administration, such nonequilibrium surface lipid compositions may be expected to influence serum protein adsorption to the particle surface, possibly influencing tissue specificity.

CONCLUSIONS

The major finding of this investigation is that LNP-siRNA systems formed by rapid mixing-ethanol dilution processes do not show evidence of inverted micellar structures containing siRNA dispersed in a "currant bun" pattern in the LNP interior. Rather, the siRNA is associated with closely apposed lipid bilayers sandwiching siRNA molecules that segregate toward the periphery of the LNP. Excess ionizable cationic lipid forms an amorphous lipid core that likely corresponds to an oil-droplet phase that contains a limited amount of cholesterol. These findings suggest that the proportions of different lipid species in optimized LNP-siRNA systems may vary according to the particular ionizable cationic lipid employed. For example, for the KC2 lipid employed here, the limited solubility of cholesterol in the hydrophobic core suggests that the cholesterol content should be reduced to achieve more stable systems. Alternatively, increasing the amount of DSPC may be expected to result in enhanced stability and possibly enhanced activity of smaller LNP systems. Previous work has shown that smaller LNP-siRNA systems are less potent than larger systems.[17]

MATERIALS AND METHODS

Materials. The lipid 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine (DSPC), 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphorylcholine (POPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000] (ammonium salt) (PEG-DSPE), and 1,2-di-*O*-octadecenyl-3-trimethylammonium propane (chloride salt) (DOTMA) were purchased from Avanti Polar Lipids (Alabaster, AL). The ionizable cationic lipid 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA) was synthesized by Biofine International (Vancouver, BC). The ionizable cationic lipid 1,2-dioleyloxy-3-dimethylamino-propane (DODMA) was purchased from Cayman Chemical (Ann Arbor, MI). Cholesterol and glyceryl trioleate (triolein) were purchased from Sigma-Aldrich (St. Louis, MO). (*R*)-2,3-bis(tetradecyloxy)propyl-1-(methoxy polyethylene glycol 2000) carbamate (PEG-DMG) was
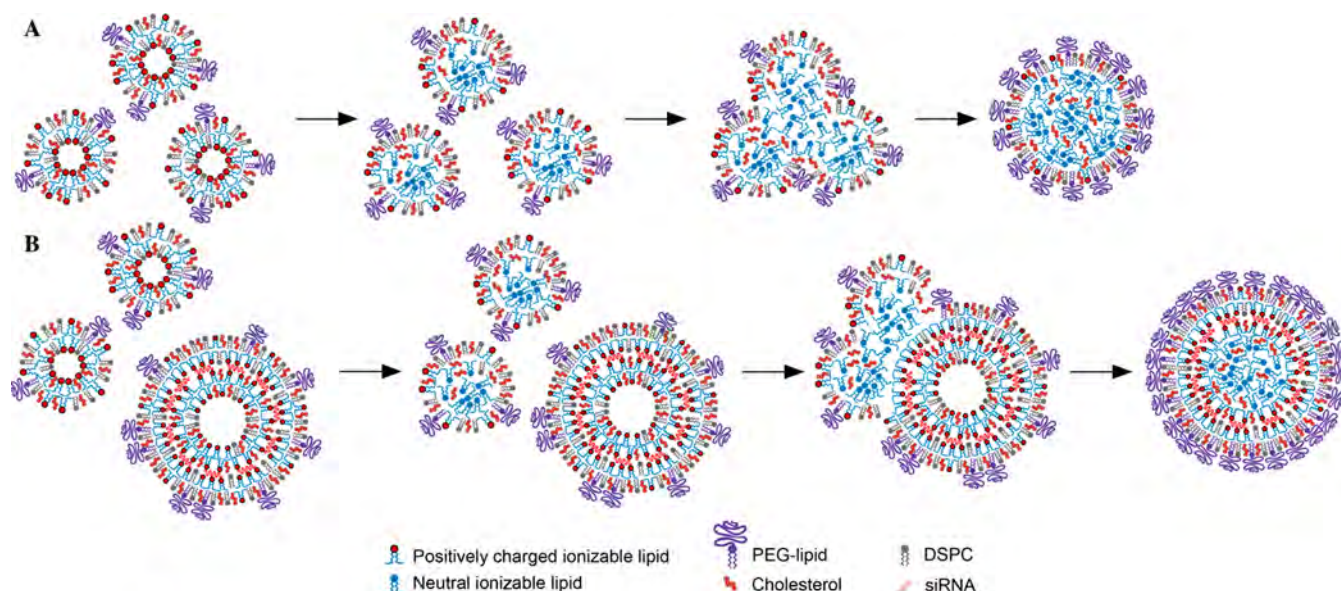
Figure 6. Proposed mechanism of formation and structure of LNP prepared in the absence and presence of siRNA. **(A)** In the absence of siRNA, the LNP lipid dispersions form small unilamellar vesicles on rapid mixing at pH 4. As the pH is raised (dialysis against PBS pH 7.4), an increasing proportion of the ionizable cationic lipids adopts a neutral form, thus decreasing intervesicle electrostatic repulsion, destabilizing the bilayer structure, and engendering vesicle fusion. As the vesicles fuse, PEG-lipid, DSPC, and cholesterol (equimolar with DSPC) partition to the outer monolayer of the increasingly large LNP, whereas neutral KC2 partitions to the LNP interior forming an oil droplet phase in the center of the LNP. Equilibrium is achieved when the concentration of PEG-lipid in the outer monolayer is sufficiently high to inhibit further inter-LNP fusion. Note that this equilibrium size may well be considerably smaller than the equilibrium size dictated by the DSPC-cholesterol content. **(B)** In the presence of siRNA, the initial event is formation of small vesicles which contain siRNA between closely apposed lipid monolayers. As the pH is raised, neutralization of the ionizable lipid induces fusion between various particles similar to the case of empty LNPs. This process is limited by phase separation of PEG-lipid, and possibly DSPC/cholesterol, from the complexes. It is proposed that these lipids are deposited in a surface monolayer that inhibits further fusion. It should be noted that the presence of high levels of ethanol (at least 25% by volume) results in high exchange rates for individual lipid molecules (with the exception of the cationic lipid complexed to siRNA), resulting in rapid formation of equilibrium structures. Note also that the DSPC/cholesterol must sequester, at least in part, to the outer monolayer and stabilize smaller structures at pH 4, as very large micron-size systems are observed for systems containing 1.5 mol % PEG-lipid and no DSPC or cholesterol (see Supporting Figure 6C). As the pH is raised, the situation is much the same as for the LNP in the absence of siRNA; the increasing conversion of the ionizable lipid to the neutral form favors further fusion and deposition in the interior core of the LNP.

synthesized as previously described.[24] The structures of the lipids used are shown in Supporting Figure 8. TEM grids were purchased from Ted Pella, Inc. (Redding, CA). siRNA against firefly luciferase[25] was purchased from IDT (Coralville, IA).

Preparation of Empty LNPs. Previous studies on the morphology of LNP-siRNA systems were conducted with particles generated using a staggered herringbone micromixer (SHM) made of polydimethylsiloxane (PDMS) provided by Precision Nanosystems Inc. (Vancouver, BC).[7,8,10] Here we show both mixing techniques (T-junction mixer[12,13,15] and SHM) generated empty LNPs and LNP-siRNA with similar morphology as observed by cryo-TEM (Supporting Figure 9). Briefly, component lipids (ionizable cationic lipids, DSPC, cholesterol, and PEG-DMG or PEG-DSPE) or emulsion lipids (POPC, triolein) were dissolved in ethanol at appropriate ratios to a final concentration of 15 mM total lipid. The appropriate aqueous and organic solutions were mixed using a T-junction mixer[12,13,15] at a flow rate ratio of 3:1 (v/v; respectively) and a total flow rate of 20 mL/min. The resultant mixture was dialyzed directly against 1000-fold volume of appropriate buffer.

Preparation of LNPs Containing siRNA. LNPs containing siRNA were prepared as previously described.[13] Briefly, component lipids (ionizable cationic lipids, DSPC, cholesterol, and PEG-DMG) were dissolved in ethanol at appropriate ratios to a final concentration of 15 mM total lipid. Nucleic acids were dissolved in 25 mM sodium acetate pH 4 buffer. The aqueous and organic solutions were mixed using a T-junction mixer[12,13,15] at a flow rate ratio of 3:1 (v/v; respectively) and a total flow rate of 20 mL/min. The resultant mixture was dialyzed directly against 1000-fold volume of sodium acetate pH 4 buffer or PBS (pH 7.4) overnight.

Cryogenic Transmission Electron Microscopy. LNPs were concentrated to a final concentration of 15−25 mg/mL of total lipid.

2−4 μL of LNP suspension was added to glow-discharged copper grids and plunge-frozen using a FEI Mark IV Vitrobot (FEI, Hillsboro, OR) to generate vitreous ice. Grids were stored in liquid nitrogen until imaged. All samples were imaged with a 200 kV instrument unless otherwise specified.

For 200 kV Imaging. Grids were moved into a Gatan 70° cryo-tilt transfer system pre-equilibrated to at least −180 °C and subsequently inserted into the microscope. An FEI LaB6 G2 TEM (FEI, Hillsboro, OR) operating at 200 kV under low-dose conditions was used to image all samples. A bottom-mount FEI Eagle 4K CCD camera was used to capture all images. All samples (unless otherwise stated) were imaged at a 55,000× magnification with a nominal under-focus of 1−2 μm to enhance contrast. Sample preparation and imaging were performed at the UBC Bioimaging Facility (Vancouver, BC).

For 300 kV Imaging. Grids were transferred to an autoloader-equipped FEI Titan Krios (FEI, Hillsboro, OR) operating at 300 kV with a Falcon III direct electron detector. All samples (unless otherwise stated) were imaged at a 47,000× magnification with a nominal under-focus of 1−2 μm to enhance contrast. Sample imaging was performed at the UBC Life Sciences Centre (Vancouver, BC).

Analysis of LNPs. Particle size analysis of LNPs in PBS was carried out using a Malvern Zetasizer (Worcestershire, UK). Cryo-TEM micrographs obtained for each sample were characterized for particle size (as compared by diameter to the scale bar), performed by manual counting of 150 LNPs. Such an approach has been shown to closely correlate with the number-weighted average produced by dynamic light scattering.[8,23] Similarly, morphology of LNPs was quantified manually. Lipid concentrations were measured using the Cholesterol E Total-Cholesterol assay (Wako Diagnostics, Richmond, VA).

Solubility of Cholesterol in KC2 oil. Cholesterol (40 mg) was transferred to a glass vial containing 200 mg of KC2 oil. The vials were then sonicated in a bath sonicator for 60 min at room temperature with intermittent vortex-mixing. The resulting mixture was then centrifuged for 30 min at 17,000×g at room temperature. The supernatant was collected, and 10.6 mg was suspended in a 1 mL of isopropanol:methanol (1:1 v/v). The concentration of cholesterol was determined by ultrahigh-pressure liquid chromatography (UPLC) on a Waters Acquity H-Class UPLC System equipped with a BEH C18 column (1.7 μm, 2.1 Å, ~100 mm) and a photodiode array detector. Separation was achieved at a flow rate of 0.5 mL/min, with a mobile phase consisting of a linear gradient of solvent A (1:1 methanol-acetonitrile mixture) and B (water) from 30:70 to 100:0, respectively, over 6 min at a column temperature of 55 °C. The absorbance at 207 nm was measured, and the cholesterol concentration was determined using calibration curves.

Small Angle X-ray Scattering. Small angle X-ray scattering (SAXS) experiments were conducted on the SAXSLAB Ganesha 300XL SAXS system at 4D Laboratories (SFU, Burnaby, BC). The sample to detector distance was adjustable across 1.4 m to allow measurements from $q = 0.0025$ Å^{-1} to $q = 2.8$ Å^{-1}. The X-ray beam has a wavelength of 0.154 nm generated by a Cu-Kα X-ray source. Concentrated LNP suspensions were loaded into quartz capillary tubes purchased from Charles Supper Company (Natick, MA) which are approximately 80 mm long, 1.5 mm in diameter, and 0.01 mm thick. After transfer, the tubes were sealed using capillary wax. Samples were loaded into a temperature-controlled Linkam heater stage which maintained a constant temperature of 22.7 °C throughout all experiments.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.8b01516.

Empty LNP morphology at high KC2 contents, morphology of LNP containing DODMA, quantification of cholesterol solubility in KC2, empty DSPC vesicles in different buffers, SAXS patterns for full q range, LNP morphology with high KC2 and siRNA, cryo-TEM of empty and loaded LNP-siRNA at pH 4 by 300 kV imaging, structures of lipids investigated in the study, and a comparison of T-tube and microfluidic LNP formulation methods (PDF)

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The authors declare no competing financial interest.

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JOINT APPENDIX 37

vaccines

MDPI

Review

Nanomaterial Delivery Systems for mRNA Vaccines

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Abstract: The recent success of mRNA vaccines in SARS-CoV-2 clinical trials is in part due to the development of lipid nanoparticle delivery systems that not only efficiently express the mRNA-encoded immunogen after intramuscular injection, but also play roles as adjuvants and in vaccine reactogenicity. We present an overview of mRNA delivery systems and then focus on the lipid nanoparticles used in the current SARS-CoV-2 vaccine clinical trials. The review concludes with an analysis of the determinants of the performance of lipid nanoparticles in mRNA vaccines.

Keywords: mRNA; lipid nanoparticle; ionizable lipid; vaccine; SARS-CoV-2

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1. Introduction

mRNA vaccines have been propelled to the center stage of the biotechnology and pharmaceutical industry by the COVID-19 pandemic. There are eight ongoing human trials for mRNA vaccines led by BioNTech/Pfizer, Moderna, CureVac, Sanofi/TranslateBio, Arcturus/Duke-NUS Medical School in Singapore, Imperial College London, Chulalongkorn University in Thailand, and Providence Therapeutics [1]. Remarkably, two of these trials have announced interim phase 3 trial results that report an efficacy providing a greater than 94% reduction in SARS-CoV-2 infection after 2 doses of 30 µg or 100 µg of an mRNA sequence encoding for a spike protein immunogen, delivered in a lipid nanoparticle [2,3]. The rapidity of vaccine development also exceeded expectations, with these results occurring only 10 months after the SARS-CoV-2 sequence was made publicly available. This success is a testament not only to the ability of the biotech and pharmaceutical industry to respond to an urgent and unmet global need, but also to the inherent capabilities of mRNA as a pharmaceutical modality, in this case a prophylactic vaccine. The purpose of this review is to overview the development of delivery systems for mRNA and then to summarize the preclinical and clinical findings of the SARS-CoV-2 mRNA vaccines and relate them to characteristics of the delivery system that contribute to their success. Several excellent reviews of mRNA delivery systems for vaccines and therapeutics that predate COVID-19 have been recently published [4–16].

Messenger RNA therapeutics have many advantages and several challenges compared to other pharmaceutical modalities, including small molecules, DNA, oligonucleotides, viral systems and proteins, including antibodies. The ability to mediate both stimulatory and inhibitory modes of action compared to oligonucleotides and most small molecule drug targets, and to express or replace defective proteins, expands the scope of potential indications for their use. Compared to DNA, mRNA only needs access to the cytoplasmic

ribosomal translation machinery rather than the nucleus and does not risk genomic integration. Compared to both proteins and viral systems, mRNA manufacturing is cell-free, faster, and the protein product bears native glycosylation and conformational properties. When combined with a lipid nanoparticle (LNP) delivery system, the nanostructural properties of the mRNA LNP also bear a resemblance to viral systems and circulating endogenous, lipid-containing chylomicrons in terms of their size, lipid envelope and, for viral systems, the internal genomic material that contributes to their application as delivery vehicles for vaccines and other therapeutics [17].

The challenges inherent to the mRNA platform are its intrinsic immunogenicity, susceptibility to enzymatic degradation, and almost negligible levels of cell uptake of naked mRNA. The innate immunogenicity of mRNA is due to the cellular detection of single- and double-stranded RNA by toll like receptors (TLRs)), helicase receptors, including retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and others [18,19], which then signal through NF-κB and interferon (IFN) regulatory factors IRF3 and IRF7, which translocate to the nucleus to bind to the type I IFN gene promoter, inducing expression of type I IFNs (IFN-α and IFN-β), accompanied by proinflammatory cytokines, such as tumor necrosis factor-a (TNF-α), IL-6 and IL-12 [20]. The secreted interferons signal through their receptors and the JAK/STAT pathway in the same cell and adjacent cells to activate more than 300 IFN-stimulated genes, including the protein kinase PKR, as a general viral defense mechanism. Although this activation could be beneficial for mounting an immune response to mRNA vaccines, one immediate effect is the downregulation of translation through PKR phosphorylation of eIF2a, which impairs eIF2 activity, inhibiting mRNA translation and thus the protein synthesis of the immunogen [21]. The primary means of abrogating this innate immune response is by substituting naturally occurring nucleosides such as 1-methylpseudouridine [22] and other nucleosides present in transfer and ribosomal RNA (but not typically in mRNA) into the mRNA sequence, which then renders it undetectable via these innate immune sensors [23,24]. This nucleoside-modified immunosilencing mRNA platform is the basis of the mRNA technologies that have recently shown >94% efficacy in the BioNTech/Pfizer and Moderna SARS-CoV-2 vaccine trials, building upon previous trials for other pathogens, which are described in detail below. A second approach pursued by CureVac is sequence engineering involving codon optimization and uridine depletion [25] since TLR7 and TLR8 primarily recognize GU-rich single-stranded RNA sequences [26]. The second challenge for mRNA therapeutics is its susceptibility to nucleases, exemplified by a half-life in serum <5 min [27]. Although chemical modifications of siRNA are highly successful in improving stability and lowering immunogenicity [28], to date, they have not been successful for mRNA due to the sensitivity of the translation machinery to these modifications [29]. The third challenge for mRNA is the lack of cell uptake of naked mRNA in most cell types [30], with the exception of immature dendritic cells [31]. These last two challenges are addressed by the incorporation of a nucleoside-modified or sequence-engineered mRNA into a delivery system that both protects the mRNA from enzymatic attack and facilitates cellular uptake. For example, incorporation into lipid nanoparticles protects the mRNA from enzymatic attack and enhances cell uptake and expression by up to 1000-fold compared to naked mRNA when administered in animal models [32,33].

Therapeutic mRNA is produced by in vitro transcription (IVT) from a plasmid DNA backbone to produce a full length message bearing a 5′ cap, a 5′ untranslated sequence (UTR), the open reading frame coding for the protein of interest, the 3′UTR and a polyA tail [4]. The natural eukaryotic 5′ cap (cap0) is an inverted 7-methyl guanosine (m7G) linked to the first nucleotide of the mRNA by a 5′ to 5′ triphosphate. Cap0 protects endogenous mRNA from nuclease attack, is involved in nuclear export and binds to translation initiation factor 4 to start protein translation. Two additional 5′ caps have been identified (cap1 and cap2) that contain additional methyl groups on the second or third ribonucleotide and are less immunogenic than cap0 (and therefore preferred) [34]. A commonly used current capping method involves a co-transcriptional capping process resulting in cap1,

which possesses high translation and low immunogenicity [35]. The 5′UTR is involved in translation initiation and can contain a Kozak sequence as well as an internal ribosomal entry site for cap-independent translation [36]. The open reading frame is followed by the 3′UTR, which influences mRNA stability and durability of protein expression. The polyA tail is encoded at around 100 residues and helps initiate translation and delay degradation. IVT production of mRNA needs to be followed by careful purification to remove DNA and double-stranded RNA contaminants, which are immunogenic [37,38]. The mRNAs described above can be nucleoside modified or sequence engineered without nucleoside modification, but are not capable of self-replication. Self-amplifying mRNA (samRNA) capable of replication are also being tested in clinical trials for SARS-CoV-2 and are longer ~10 kb sequences since they contain four additionally encoded nonstructural genes, including an RNA-dependent RNA polymerase, which result in self-replication inside cells but do not produce an infectious particle since they lack structural genes [39]. samRNAs cannot be nucleoside modified since these modifications interfere with self-amplification. Due to the amplification process, samRNAs typically use lower doses (1–10 μg) in the current COVID-19 clinical trials compared to 30–100 μg for non-amplifying mRNA. Interestingly, all of the above categories of mRNA vaccines are currently being tested in human clinical trials for SARS-CoV-2 and are summarized in Table 1. All mRNA delivery systems in these clinical trials are lipid nanoparticles. The exact composition of the Pfizer-BioNTech LNP [40] and Moderna LNP [41] have been publicly disclosed, while some others have not. The others are all most likely similar to the Alnylam Onpattro™ product (described further below) but with a proprietary ionizable lipid, as is the case for those that are disclosed. Although the specific ionizable lipid used may not be known in all cases, its general class can be understood from journal and patent publications and is indicated in Table 1.

Table 1. Current human trials for SARS-CoV-2 using mRNA lipid nanoparticles. All mRNA vaccines in SARS-CoV-2 clinical trials use a lipid nanoparticle for delivery. The identity and composition of each has not been publicly disclosed, so their probable class (shown below) is based on the available literature and patent citations.

Company	mRNA Type	Immunogen	mRNA Dose (μg)	Confirmed or Probable LNP Class	Publications
Moderna	nucleoside modified mRNA	membrane bound prefusion stabilized spike	100	Lipid H [42] confirmed in [41]	[43–46]
BioNTech Pfizer	nucleoside modified mRNA	membrane bound prefusion stabilized spike	30	Acuitas ALC-0315 [47] confirmed in [40]	[48–51]
CureVac	unmodified mRNA	membrane bound prefusion stabilized spike	12	Acuitas ALC-0315 [47]	[52,53]
TranslateBio Sanofi	unmodified mRNA	prefusion stabilized double mutant spike	7.5	ICE [54] or Cysteine [55]	[56]
Arcturus	self-amplifying mRNA	full length spike	1–10	Lipid 2,2 (8,8) 4C CH$_3$ [57]	[58]
Imperial College	self-amplifying mRNA	membrane bound prefusion stabilized spike	1–10	Acuitas A9 [59]	[60]
Chulalongkorn	nucleoside modified mRNA	secreted wild type spike	Not available	Genevant CL1 [61]	NA

Prior to COVID-19, mRNA vaccines were used in preclinical and clinical studies for infectious diseases including influenza, zika, HIV, Ebola, rabies, chikungunya, malaria, genital herpes, toxoplasma gondii, and others. These studies are summarized in a number of excellent recent reviews [4,6,16,39].

2. Early Delivery Systems for mRNA Vaccines

Protamine, a mixture of small arginine-rich cationic proteins, has been used to form complexes with mRNA that improved transfection compared to naked mRNA [62]. Later, a mixture of free mRNA with protamine-complexed mRNA was introduced [63] since protamine-complexed mRNA partly inhibited protein expression [64]. Dynamic light scattering indicated that free mRNA have a size near 50 nm, while the protamine/mRNA

complexes were in the 250–350 nm range [63]. This approach was pursued by CureVac for a rabies vaccine candidate, CV7201, a lyophilized, temperature-stable non-modified mRNA composed of free and protamine-complexed mRNA encoding the rabies virus glycoprotein (RABV-G) [65]. In Balb/c mice, two doses of 10 µg and higher induced neutralizing titers greater than the WHO threshold of protection and administration of an 80 µg dose twice was protective against a lethal intracerebral challenge [66]. In a phase 1 human trial using doses 80–640 µg applied through intradermal and intramuscular routes, only a subgroup of participants who received three 80–400 ug doses using a particular injector device achieved the WHO neutralization titer threshold [67]. A serious adverse event (Bell's Palsy) occurred for one participant out of 101 at the highest dose and 5% of all participants experienced a solicited severe adverse event. The overall rate of all adverse events was high, with 97% experiencing injection site reactions and 78% a systemic adverse event. Given this suboptimal delivery with protamine complexed mRNA, CureVac adopted a lipid nanoparticle delivery system from Acuitas [47,68] and demonstrated greatly improved neutralizing titers at a 20-fold lower dose of 0.5 µg (vs. 10 µg for protamine complexed mRNA) in Balb/c mice and at a 10 µg dose in non-human primates [69]. Activation of T cell responses and the presence of IL-6 and TNF in the draining lymph nodes and injection sites indicated the role of the LNP in mediating the positive immune response. A clinical trial has been initiated (NCT03713086), with interim results expected to be reported in 2021.

A cationic nanoemulsion (CNE) was developed for mRNA delivery by combining the cationic lipid DOTAP with a commercial adjuvant (MF59) containing squalene, sorbitan trioleate, and polysorbate 80 in a citrate buffer of pH 6.5 [70]. The combined use of a self-amplifying mRNA encoding for respiratory syncytial virus glycoprotein (RSV-f) with an NP amine (from DOTAP) to phosphate ratio (of mRNA) of 7 resulted in an average 129-nm sized nanoparticle. One advantage of this approach is the ability to store CNE and mRNA separately and combine them only at the time of use. A 15-µg dose administered twice in Balb/c mice elicited neutralizing titers above that of an adjuvanted subunit vaccine. Detectable neutralization titers and T cell responses in non-human primates were achieved with two doses of 75 µg. Building on this concept, a separate group created a Nanostructured Lipid Carrier (NLC), which is a hybrid between a CNE and a lipid nanoparticle, consisting of a liquid oil phase, such as squalene, with a solid-phase lipid composed of a saturated triglyceride [71]. NLCs containing a self-amplifying mRNA encoding for a sika immunogen had a particle size of 40 nm and an NP ratio of 15 and were capable of generating protective neutralizing titers in C57BL/6 mice after a single injection of a dose as low as 0.1 µg or 0.01 µg.

3. Polymers for mRNA Delivery

Cationic polymers have been widely used for nucleic acid delivery for several decades, including for example poly(L-lysine), polyethylenimine (PEI), DEAE-dextran, poly(β-amino esters) (PBAE) and chitosan. In their simplest format, cationic polymers are mixed in excess with nucleic acid to form electrostatically bound cationic polyplexes. Although many polymers have been developed, they are not as advanced as lipid nanoparticles for nucleic acid delivery and the number of animal studies applying them successfully to vaccines is limited. PBAEs were co-formulated with polyethylene glycol (PEG)-lipids to form mRNA/PBAE/PEG–lipid nanoparticles that were capable of the functional delivery of mRNA to the lungs after intravenous administration in mice [72]. A biodegradable polymer, poly(amine-co-ester) (PACE) terpolymer, has been examined for mRNA delivery using erythropoietin as a reporter post-IV administration for gene delivery [73]. By controlling the molecular weight and end group chemistry, a 10 kDa member of the PACE family achieved the same in vitro transfection efficiency as TransIT, a potent but toxic colloidally unstable and large-sized commercial reference. In vivo expression of EPO at 20 µg IV was fivefold more potent than TransIT. Hyperbranched poly (beta amino esters) (hPBAEs) were synthesized for mRNA delivery to the lung by inhalation. hPBAE mRNA polyplexes were 137 nm in size and were able to transfect 25% of the lung endothelium when nebulized and

inhaled in mice without evident toxicity and with expression levels 10-fold that of branched PEI [74]. A disulfide-linked poly(amido amine), pABOL, was synthesized at molecular weights ranging from 8 kDa to 167 kDa and was able to form polydisperse nanocomplexes near 100 nm in size [75]. In vivo luciferase expression of these polyplexes using a self-amplifying mRNA reporter was similar to that of PEI after intramuscular administration. When delivered to mice with a hemagglutinin (HA) influenza immunogen in a prime-boost design, neutralizing titers were highest for the low molecular weight 8 kDa pABOL and exceeded those of PEI. The 8 kDa pABOL delivering 1 μg HA of self-amplifying mRNA was also partly protective against a lethal influenza challenge, preventing death but not preventing significant weight loss. This pABOL system was considered for the delivery of a self-amplifying mRNA immunogen for SARS-CoV-2 by the group at Imperial College London; however, delivery of a SARS-CoV-2 immunogen with pABOL was 1000X less potent than delivery of the same immunogen with an optimized lipid nanoparticle from Acuitas [59]. In total, 1μg of self-amplifying RNA in pABOL generated the same binding antibody and neutralization titers as 0.001 μg in an optimized lipid nanoparticle (Dr. Anna Blakney, personal communication). Many other polymer systems are capable of delivering mRNA in vitro or in vivo but remain to be tested in a vaccine context [76–84].

4. Development of Lipid Nanoparticles for the Current SARS-CoV-2 Clinical Trials

The earliest transfection reagent for mRNA was the quaternized cationic DOTAP combined with ionizable and fusogenic DOPE, adopted from DNA transfection [85] for the transfection of mRNA in numerous cell types [86]. Although effective in vitro, the permanently cationic quaternized ammonium group renders these large-sized lipoplexes rapidly cleared from circulation and from generally targeting lungs, as well as exhibiting toxicity. The forerunner of today's LNP was the stabilized plasmid–lipid particle (SPLP) that was formed by combining the fusogenic ionizable DOPE with a quaternized cationic lipid, DODAC, which electrostatically bound and encapsulated plasmid DNA, which was then coated with hydrophilic PEG to stabilize it in aqueous media and limit protein and cell interactions upon administration in vivo [87]. DOPE can be protonated in the endosome after cell uptake and, since it is cone-shaped, it can form an endosomolytic ion pair with endosomal phospholipids to facilitate endosomal release, a critical event for successful delivery [17]. The SPLP was then further developed as a Stabilized Nucleic Acid Lipid Particle (SNALP) containing siRNA that included four lipids: an ionizable rather than quaternized cationic lipid, a saturated bilayer forming quaternized zwitterionic lipid, DSPC, cholesterol and a PEG–lipid [88]. In addition to electrostatically binding to the nucleic acid, the ionizable lipid in the SNALPs played the role of the fusogenic lipid and became protonated in the endosome to form a membrane-destabilizing ion pair with an endosomal phospholipid. It is now known that DSPC helps form a stable bilayer underneath the PEG surface [89]. Cholesterol plays several roles, including filling gaps in the particle, limiting LNP–protein interactions and possibly promoting membrane fusion [90]. The ionizable lipid plays a central role by being neutral at physiological pH, thus eliminating any cationic charge in circulation, but becoming protonated in the endosome at pH ~6.5 to facilitate endosomal release. The development of the first siRNA product that was clinically approved in 2018 primarily focused on optimizing the ionizable lipid and, secondarily, the PEG–lipid and the ratios of the four lipids used in the LNP, as well as the LNP assembly and manufacturing procedure. An optimal number of unsaturated bonds in the C18 tail were found to be providing a dilinoleic acid tail linked by ethers to a dimethylamine headgroup [88], in accordance with the molecular shape hypothesis [12,91]. However, the introduction of a single linker to the dilinoleic acid tail, which had an optimized number of carbons from the dimethylamine head group to the linker, resulted in the pKa of the ionizable lipid in the LNP being near 6.4 for the ionizable lipid DLin-MC3-DMA [92,93]. The last step in the optimization was to tune the mole ratios of these lipids to 50/10/38.5/1.5 for MC3/DSPC/Cholesterol/PEG–lipid. Overall, this optimization process from DLin-DMA to DLin-MC3-DMA required more than 300 ionizable lipids to be screened in thousands of

formulations and resulted in a 200-fold increase in potency and a corresponding reduction in the effective dose in order to achieve durable suppression of the target gene >80% and a therapeutic window that permitted the clinical approval of Onpattro™ in 2018 [94,95]. This MC3 formulation developed for siRNA is the basis for the subsequent development of LNPs described below (Figure 1), which are now under emergency use after being approved for the delivery of SARS-CoV-2 mRNA vaccines.

PEG-lipid   Charged ionizable lipid

Cholesterol   Neutral ionizable lipid

DSPC
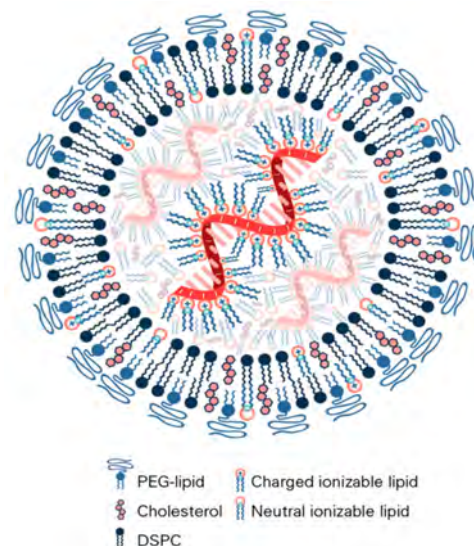
Figure 1. mRNA lipid nanoparticle structure. Recent studies using cryoelectron microscopy [96], small-angle neutron scattering and small-angle X-ray scattering [89] have shown that the mRNA Lipid nanoparticle includes low copy numbers of mRNA (1–10) and that the mRNA is bound by the ionizable lipid that occupies the central core of the LNP. The polyethylene glycol (PEG) lipid forms the surface of the lipid nanoparticle (LNP), along with DSPC, which is bilayer forming. Cholesterol and the ionizable lipid in charged and uncharged forms can be distributed throughout the LNP. Structural schematics of other delivery systems are available in a recent review [14].

Moderna carried out several preclinical [97–99] and clinical studies [97,100] using MC3 in the Onpattro formulation described above in order to deliver nucleoside-modified mRNA-encoded immunogens. MC3 was later identified [42,101] as the ionizable lipid in these studies comparing a new class of ionizable lipids to MC3. This new class includes Lipid H [42], which is the ionizable lipid SM-102 [41] in Moderna's SARS-CoV-2 product mRNA-1273 (Table 2). Using a nucleoside-modified mRNA-encoded immunogen for the Zika virus, the MC3 LNP was capable of protecting immunocompromised mice lacking type I and II interferon (IFN) signaling against a lethal challenge with one 10 µg dose or two 2 µg doses in a prime-boost design [99]. Similar results were obtained in immunocompetent mice pre-administered with an anti-ifnar1 blocking antibody to create a lethal model. In a series of influenza studies delivering nucleoside-modified mRNA-encoding hemagglutinin (HA) immunogens, the MC3 LNP delivered intradermally was capable of fully protecting mice against a lethal challenge with a single dose as low 0.4 µg, although post-challenge weight loss occurred even when up to 10 µg of a single dose was administered [97]. A single dose of 50 µg or 100 µg produced high HAI (hemagglutination inhibition assay) titers in ferrets, as did two doses of 200 or 400 µg in non-human primates. In a small number (23) of human subjects who received 100 µg doses, all had HAI titers >40 (the WHO correlate of protection) that were more than fourfold above the baseline at the beginning of the study. In a larger phase 1 trial using these same MC3 LNPs delivering two distinct nucleoside-modified mRNA-encoded HA immunogens, intramuscular injection of 100 µg of the H10N8 immunogen resulted in 100% of the 23 subjects having HAI titers >40 [100]. Although no life-threatening adverse events occurred, 3 of these 23 subjects experienced severe grade 3 adverse events. A planned 400 µg dose was discontinued after two of three

subjects experienced grade 3 adverse events, which met the study pause rules. At lower doses, the frequency and severity of adverse events diminished, although nearly every subject experienced at least one adverse event. These studies were promising, but also highlighted the relatively narrow therapeutic window to obtain protective immunizations at doses that do not cause a problematic number of adverse events. This is reminiscent of the narrow therapeutic window of the MC3 precursor, DLin-DMA, which needed an improved potency in order to lower the dose and still achieve efficacious gene knockdown.

Table 2. Ionizable lipids used in lipid nanoparticles. A key feature of the ionizable lipids used in lipid nanoparticles is that the pKa of the ionizable lipid in the LNP, as measured by the TNS dye-binding assay, should be in the range of 6–7. The theoretically calculated pKa of most of the ionizable groups is in the range of 8–9.5, as shown below on the nitrogen atoms, using commercial software that theoretically estimates these values in aqueous media. The 2–3 point drop in pKa from the theoretical value to the TNS value is due to the much higher energy of solvation of protons in the lipid phase, creating a pH increase of 2–3 points in the lipid compared to the aqueous phase, where pH is measured during the TNS assay [102].
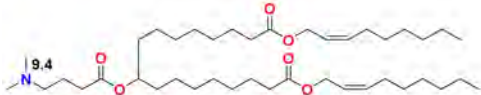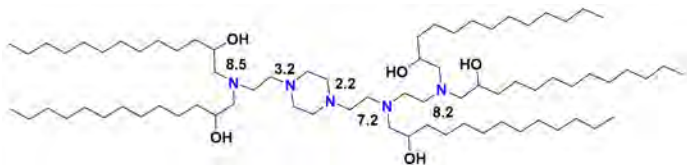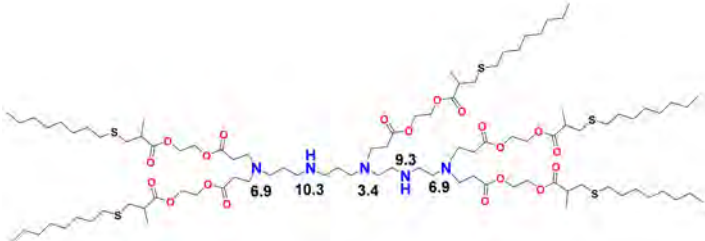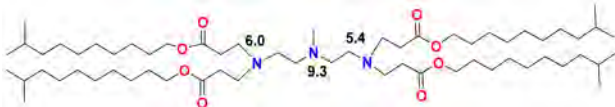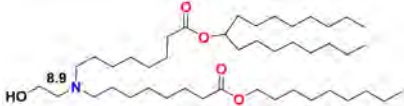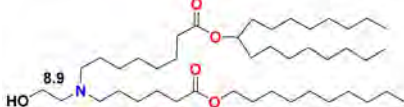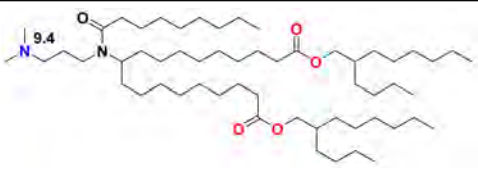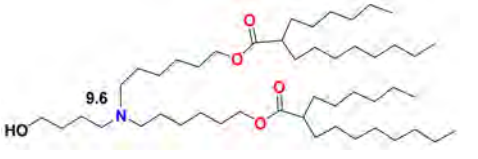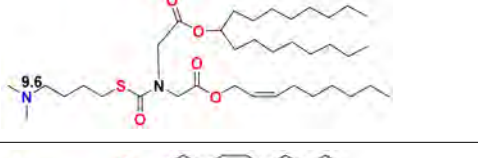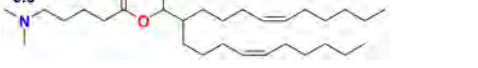
Name	Ionizable Lipid Structure and Theoretical pKas	TNS pKa
MC3 [92]		6.4
Lipid 319 [68]		6.38
C12-200 [103]		6.96
5A2-SC8 [104]		6.67
306Oi10 [105]		6.4
Moderna Lipid 5 [101]		6.56
Moderna Lipid H, SM-102 [42]		6.75

Table 2. *Cont.*

Name	Ionizable Lipid Structure and Theoretical pKas	TNS pKa
Acuitas A9 [59]		6.27
Acuitas ALC-0315 [47]		6.09
Arcturus Lipid 2,2 (8,8) 4C CH3 [57]		6.69
Genevant CL1[61]		NA

Since siRNA products require repeated dosing for chronic diseases, there was a concern that the slow degradability of the dilinoleic alkyl tail in MC3 would cause accumulation and potential toxicity with repeated dosing. A biodegradable version of MC3, Lipid 319 (Table 2), was generated by replacing one of the two double bonds in each alkyl chain with a primary ester that can be easily degraded by esterases in vivo [68]. A half-life of less than an hour in the liver was noted for Lipid 319, while it maintained a gene silencing efficiency in the liver that was similar to MC3. The degradation products were confirmed in vivo, as well as their secretion and the nontoxic nature of Lipid 319. This study of Lipid 319 is cited in the preclinical and clinical studies for SARS-CoV-2 as representing the Acuitas LNP class used in the BioNTech [49] and CureVac [53,69] products, although the Acuitas LNP delivering the self-amplifying RNA in the Imperial College London trial [60] is cited as having been contained in a more recent patent application [59], represented here by Lipid A9 from Acuitas (Table 2). Recently, the identity of the Acuitas ionizable lipid in BioNTech's approved BNT162b2 was disclosed [40] as ALC-0315 (Table 2). An important aspect of these LNPs is that they were developed by screening mRNA expression in the liver following IV administration and may not yet be fully optimized for the intramuscular administration of mRNA-based vaccines.

Moderna recently developed a new class of ionizable lipids to replace MC3, primarily due to the above-mentioned concerns related to the slow degradability of MC3, but also with the effort of increasing their potency by enabling greater branching than the dilinoleic MC3 alkyl tail [42,101]. This new class of lipids has an ethanolamine ionizable head group, connected to both a single saturated tail containing a primary degradable ester—like that of Maier 2013—and a second saturated tail that branches after seven carbons into two saturated C8 tails using a less degradable secondary ester, as in Lipid 5 [101] (Table 2), optimized for IV administration to the liver, and a similar Lipid H [42] or SM-102, found to be optimal for the intramuscular (IM) administration of vaccines. Increased branching is a common feature pursued by Acuitas, as Lipid A9 has a total of five branched chains [59] (Table 2) vs. three for the Moderna LNPs. Increased branching is believed to create an ionizable lipid with a more cone-shaped structure, so that—when paired with the anionic phospholipid in the endosome—a greater membrane-disrupting ability will occur, following the molecular shape hypothesis outlined several decades ago [12,91]. When administered IV, Lipid 5 was not detectable in the liver at 24 h, while MC3 was present

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in the liver at 71% of its initial dose, verifying the degradability of Lipid 5. Lipid 5 was three-fold more potent than MC3 in mice for luciferase expression and five-fold more potent in non-human primates for hEPO after IV administration. These increases in potency were consistent with and possibly caused by an increase in endosomal release, with up to 15% of the mRNA in the cell being released from the endosome for Lipid 5 versus 2.5% for MC3, the latter being similar to that previously measured for MC3 using siRNA [106]. However, cell uptake in these endosomal release experiments was fourfold higher for MC3 vs. Lipid 5 so that absolute amounts of released mRNA in the cytoplasm were similar for these two LNPs. The same ionizable lipid library was examined in intramuscular administration for vaccines and was similarly found to be degradable and quickly eliminated due to the primary ester and generally to have a 3–6 fold increase in potency in terms of protein expression or immunogenicity compared to MC3 for an influenza nucleoside-modified mRNA encoded immunogen in mice, although immunogenicity in non-human primates was identical to MC3 at a 5 μg prime-boost dose [42]. Lipid H or SM-102 (Table 2) was identified as the optimal candidate and structurally only differs from Lipid 5, identified as optimal for IV administration, by a two-carbon displacement of the primary ester. The pKa of Lipid 5 LNP was 6.56, while that of the Lipid H LNP was 6.68, suggesting that a slight increase in pKa may be beneficial for IM vs. IV administration, although this difference is within the variability of the assay. Histological examination of muscle injection sites in rats indicated that Lipid H LNPs attracted less of a neutrophil- and macrophage-enriched inflammatory infiltrate compared to MC3, which may reduce injection site reactogenicity in human trials [42].

5. mRNA Lipid Nanoparticles in the Current SARS-CoV-2 Clinical Trials

5.1. BioNTech/Pfizer

Acuitas ALC-0315 (Table 2) combined with DSPC, cholesterol and a PEG–lipid is the delivery system in the SARS-COV-2 trials of BioNTech [40]. CureVac and Imperial College London may also use ALC-0315, or possibly A9 (Table 2). BioNTech began developing its SARS-CoV-2 vaccine with four mRNA-encoded immunogens, two of which were nucleoside modified, one unmodified and one self-amplifying. Reports are available for the two nucleoside-modified mRNAs: BNT162b1 is a short ~1 kb sequence encoding the receptor-binding domain of the spike protein, modified by a foldon trimerization domain to increase immunogenicity by multivalent display. The longer 4.3 kb BNT162b2 encodes a diproline-stabilized, full-length, membrane-bound spike protein. BNT162b2 received EU and US emergency approval recently. In a preclinical study, binding antibodies and neutralization titers in mice were detectable after a single dose of 0.2, 1, and 5μg of BNT162b2, increasing by an order of magnitude from the lowest to the highest dose and eliciting strong antigen-specific Th1 IFNγ and IL-2 responses in CD4+ and CD8+ splenocytes with very low levels of Th2 cytokines [49]. Draining lymph nodes also contained high numbers of germinal center B cells and elevated counts of CD4+ and CD8+ T follicular helper (Tfh) cells, which were previously identified as partly induced by the LNP alone in mRNA LNP vaccines [33]. In non-human primates, prime-boost doses of either 30 μg or 100 μg elicited binding antibody and neutralization titers that were more than 10 fold those of a human convalescent panel and a strongly Th1-biased T cell response that is believed to be important to protect against vaccine-associated enhanced respiratory disease [107]. In a limited number (6) of challenged rhesus macaques, two doses of 100 μg rendered undetectable viral titers in bronchoalveolar lavage and from nasal swabs. A phase 1 clinical trial for the smaller mRNA-encoded immunogen BNT162b1 planned 10, 30 and 100 μg doses on day 1 and day 21. The intermediate dose of 30 μg induced antibody binding and neutralization titers that were 30-fold and threefold higher than those of a human convalescent panel, respectively. The 100 μg dose was not administered for the boost due to the presence of severe injection site pain after the first dose. Injection site pain was reported by 100% of subjects with the 30 μg boost, but at mild or moderate severity. Following the second vaccination at the 30 μg dose, nearly all subjects experienced mild or

moderate systemic adverse events of fever, chills or fatigue. This trial also demonstrated strong Th1-biased T cell responses from peripheral blood mononuclear cells [50]. A phase 2 trial compared both BNT162b1 and BNT162b2 in groups of younger (18–55 y) and older (65–85 y) subjects [51]. Binding and neutralizing antibody titers were slightly lower in the older subjects, but still exceeded those in a convalescent panel. The severity of adverse reactions was also reduced in the older versus younger subjects. A significant reduction by ~twofold in the frequency of systemic adverse events (fever, chills, fatigue) was found in BNT162b2 versus BNT162b1. It was this increase in the tolerability of BNT162b2 that drove its selection for the phase 3 trial, where a 94% effectiveness was recently announced, since 162 COVID-19 cases occurred in the placebo arm, while only 8 cases were found in the vaccinated group that received two 30 µg doses of BNT162b2 [3].

5.2. Moderna

The nucleoside-modified mRNA encoded immunogen in Moderna's studies is a transmembrane-anchored diproline-stabilized prefusion spike with a native furin cleavage site and is delivered in an LNP that follows the prototype MC3 LNP, but replaces MC3 with Lipid H (SM-102) [41,42]. This mRNA LNP (mRNA-1273) induced neutralizing antibodies in several mouse species when injected at 1 and 21 days with a 1µg dose, but not at a 0.1 µg dose [44]. The T cell response appeared to be a balanced Th1/Th2 response and viral titers in mice lungs and nasal turbinates in a mouse-adapted virus challenge model were reduced to baseline with two doses of 1 µg, but not with 0.1 µg. In rhesus macaques, 2 doses of 100 µg produced high binding and neutralizing titers and a Th1-biased response in peripheral blood that also involved a strong Tfh response [45]. Titers and T cell responses were significantly lower with two 10 µg doses. Similarly, the 100 µg dose was capable of reducing viral titers in bronchoalveolar lavages and nasal swabs to baseline, while 10 µg only did so in the lungs. In a phase 1 study with 15 patients per group receiving 2 doses of 25, 100 or 250 µg, separated by 4 weeks, binding and neutralization titers were ~10-fold higher than convalescent for the 100 µg dose, and about equivalent to convalescent at 25 µg [46]. Solicited adverse events were report by all subjects at the 100 µg and 250 µg doses and 3 of 14 in the 250 µg group reported severe adverse events and were discontinued. In a subsequent phase 1 study in older patients (56–71 y and above 71 y), the 25 µg and 100 µg doses were found to produce binding antibody titers above those of convalescent plasma, while neutralizing titers were equivalent at 100 µg, but lower than convalescent at 25 µg [43]. Most patients (~80%) still experienced adverse events after the second vaccination, even in the older age group. Analyses of peripheral blood showed a CD4 T cell response that was Th1 biased. The higher neutralization titers for the 100 µg dose vs. the 25 µg dose resulted in its selection for the phase 3 trial, where interim results announced a 94.5% efficacy with 90 cases of COVID-19 in the placebo group versus five in the vaccinated group [2]. An independent board conducted an interim analysis of Moderna's phase 3 trial and found that severe adverse events included fatigue in 9.7% of participants, muscle pain in 8.9%, joint pain in 5.2%, and headache in 4.5%, while, in the Pfizer/BioNTech phase 3 trial, the frequency was lower with fatigue at 3.8% and headache 2% [108].

5.3. CureVac

The CureVac mRNA LNP (CVnCoV) is a non-chemically modified, sequence-engineered mRNA encoding a diproline stabilized full-length S protein delivered in an Acuitas LNP, possibly using the ionizable lipid ALC-0315. The number of weeks between two doses was examined ranging, from 1 to 4 when using 2 µg doses in mice, where it was found that the longer intervals produced higher titers and T cell responses and a balanced Th1/Th2 response in Balb/c mice [53]. The second dose was required to produce neutralizing antibodies and two doses of 0.25 µg were insufficient to produce neutralizing antibodies. In Syrian golden hamsters, two 10 µg doses (but not 2 µg) were able to reduce viral titers in the lungs (but not nasal turbinates) to baseline. In a phase 1 clinical trial examining 2–12 µg doses, neutralizing titers reaching the levels of convalescent sera were only found at the

highest 12 μg dose, resulting in higher doses of 16 and 20 μg being included in the ongoing phase 2 trial [52]. All patients at the 12 μg dose experienced systemic adverse events after each dose, the majority being moderate and severe, while >80% experienced local injection site pain at the mild and moderate levels.

5.4. TranslateBio

Translate Bio uses a non-modified mRNA encoding a double mutant form of the diproline stabilized spike protein delivered in an LNP that is cited as being based on the ionizable lipid C12-200 [109], but may be a more recently synthesized candidate from the ICE- [110] or cysteine-based [55] ionizable lipid families. In Balb/c mice, two doses in the range of 0.2–10 μg resulted in binding and neutralization titers well above convalescent levels. In non-human primates 15, 45 and 135 μg doses all generated titers exceeding the human convalescent panel [56]. The immune response was also Th1 biased.

5.5. Arcturus

Arcturus uses a self-amplifying, full-length, unmodified mRNA encoding a pre-fusion SARS-CoV-2 full-length spike protein in an LNP that uses an ionizable lipid with a thioester to link the amine-bearing headgroup to lipid tails via two additional ester groups. Two possible ionizable lipids in this family are Lipid 10a (in Table 4 of [111]) or Lipid 2,2 (8,8) 4C CH3 (on p. 33 of [57]) (Table 2). The latter has three branches, resembling the Moderna Lipid H, but with a degradable thioester linked to the headgroup. A feature of self-amplifying mRNA was observed where luciferase reporter expression was maintained at a fairly constant level beyond one week of IM administration, while conventional mRNA expression fell quickly [58]. The vaccination alone surprisingly produced weight loss and increased clinical scores in C57BL/6 mice. Only a single dose at 2 μg or 10 μg (but not 0.2 μg) in mice was required to reach neutralization titers above 100 in a Th1-biased response with high levels of antigen-specific T cell responses. A single dose of 2 μg or 10 μg was also 100% protective in the K18-hACE2 lethal mouse challenge model, generating 100% survival with no weight loss and a reduction in lung and brain viral titers to baseline. Arcturus has completed a phase 1 clinical trial with doses from 1–10 μg and has chosen 7.5 μg for its phase 3 trial [112].

5.6. Imperial College London

Imperial College London uses a self-amplifying mRNA-encoded prefusion-stabilized spike protein delivered in an Acuitas LNP, which is described in the patent [59] represented by Lipid A9 [60] (Table 2). Remarkably high and dose-dependent antibody and neutralizing titers were obtained after two injections of doses in the range 0.01 μg to 10 μg in Balb/c mice. The response was strongly Th1 biased and the 10 and 1 μg doses produced threefold higher antigen-specific splenocyte responses compared to the lower 0.1 and 0.01 μg doses. A phase 1 clinical trial is about to start for this vaccine.

5.7. Chulalongkorn University, University of Pennsylvania

Chulalongkorn University, in collaboration with the University of Pennsylvania, is developing a native spike immunogen nucleoside-modified mRNA LNP using a Genevant LNP, likely CL1 Lipid [61]. They aim to begin phase 1 clinical trials in Q1 of 2021 and begin distribution of the vaccine in Q4 of 2021 to Thailand and seven surrounding low to moderate income countries.

5.8. Providence Therapeutics

Providence therapeutics was granted a Health Canada notice of authorization to pursue human clinical trials for the PTX-COVID-19B mRNA LNP vaccine [113]. Preclinical studies of three mRNA candidates encoding the receptor-binding domain, the full-length spike with or without a mutation in the furin cleavage site, were administered at a dose of 20 μg in C57BL6 mice following a prime-boost regimen [114]. Preclinical data using an undisclosed lipid from Genevant, possibly similar to CL1 In Table 2, showed robust neutralization titers for the full length and the furin-mutated payloads, reminiscent of the data observed in [115]. Phase 1 clinical trials are scheduled to begin in Q1 of 2021, with manufacturing and distribution of the vaccine—pending regulatory approval—in the same year.

5.9. Storage and Distribution

Most RNA LNPs made in the laboratory are stable at 4 °C for several days, but then exhibit size increases and a gradual loss of bioactivity, such as luciferase expression [116]. A size increase over time from LNP aggregation has been commonly observed in previous siRNA LNP formulations [117]. In order to stabilize mRNA LNP vaccines for storage and distribution, a frozen format has been required to date. The Moderna COVID-19 vaccine needs to be stored from −25 °C to −15 °C, but is also stable between 2 °C and 8 °C for up to 30 days and between 8 °C and 25 °C for up to 12 h [118]. The Pfizer/BioNTech COVID-19 vaccine needs to be stored from −80 °C to −60 °C and then thawed and stored from 2 °C to 8 °C for up to 5 days prior to dilution with saline before injection [119]. The dry ice temperatures required for the Pfizer vaccine are more difficult to achieve during distribution and storage than the regular freezer temperature required by the Moderna vaccine. The reasons behind these temperature differences are not obvious since both vaccines contain similar high concentrations of sucrose as a cryoprotectant. The Moderna mRNA LNPs are frozen in two buffers, Tris and acetate [41], while the Pfizer/BioNTech vaccine only uses a phosphate buffer [40]. Phosphate buffers are known to be suboptimal for freezing due to their propensity to precipitate and cause abrupt pH changes upon the onset of ice crystallization [120,121]. Lyophilization has been challenging for mRNA LNPs [116]. However, Arcturus has stated that their COVID-19 mRNA vaccine is stable in a lyophilized format, which would presumably greatly simplify distribution, although the temperature stability of this lyophilized formulation has not yet been disclosed [122].

6. Lipidoid Nanoparticles

A number of lipid-like entities, termed lipidoids, were initially developed for siRNA delivery and subsequently used for mRNA delivery. One example is C12-200 (Table 2), which was selected from a large lipidoid family due to its high efficiency in hepatocyte gene silencing via IV administration [123]. For efficient liver-directed gene silencing, C12-200 was combined with the same lipids as the MC3 Onpattro prototype, namely 50% ionizable lipid, 10% DSPC, 38.5% cholesterol and 1.5% PEG–lipid. A later study found that the C12-200 delivery efficiency for mRNA to the same liver target could be increased sevenfold by reducing the percentage of ionizable lipid to 35%, but increasing the weight ratio of ionizable lipid to nucleic acid from 5 to 10 and replacing DSPC with the fusogenic unsaturated DOPE [103]. Interestingly, this optimized formulation increased mRNA expression sevenfold, but did not change the silencing efficiency for siRNA. C12-200, in this formulation, has also been studied for mRNA-mediated protein replacement therapy in mice and nonhuman primates [124], but was seen to generate a strong inflammatory response by histology when injected subcutaneously [109]. C12-200 is a small molecule dendrimer with five alkyl chains and five nitrogen atoms, three of which appear to be protonatable, according to ionization analyses that can be performed with commercial software such as ACDLabs Percepta (Table 2). Another dendrimer lipidoid, 5A2-SC8, was identified for high siRNA delivery efficiency to the liver in a separate screening process, and also has five nitrogen atoms and five short alkyl chains [125] (Table 2). The 5A2-SC8 lipidoid

had poor efficiency for mRNA delivery unless its formulation parameters were similarly changed by lowering the ionizable lipid mole fraction to 24%, using DOPE instead of DSPC, and increasing the other lipid proportions, but, at the same time, increasing the weight ratio of 5A2-SC8 to mRNA to 20 [104]. These formulation changes appear to be needed for these dendrimer-type lipidoids to be effective mRNA delivery vehicles, possibly since they have multiprotic head groups and a dendrimer structure. Another very high molecular weight modified dendrimer was used to deliver self-amplifying mRNA encoding immunogens for influenza, Ebola and toxoplasma gondii and was shown to be protective against all three pathogens in mice after a single, very high dose of 40 μg or prime-boost 4 μg injections, which is also a high dose for replicating RNA [126]. An interesting recent finding for a series of lipidoids was that an additional single carbon branch at the terminus of each of the four alkyl chains of this small, three-nitrogen dendrimer increased the potency of liver expression more than 10-fold compared to other lipidoids in this class [105]. There was no correlation of this increased potency with the LNP pKa, but there was a correlation with the absolute fluorescence of the TNS dye at pH 5, which indicates that the amplitude of protonation in the endosome correlates to mRNA expression, presumably by facilitating endosomal release. The additional carbon branch could also be expected to produce a more cone-shaped structure and thereby more membrane disruption according to the molecular shape hypothesis [12,91].

7. Intranasal Delivery of mRNA Lipid Nanoparticles

For mRNA vaccines, the vast majority of studies and all current clinical trials have used intramuscular administration, while intradermal administration has also been studied, usually in parallel with the intramuscular route. Although not highly developed to date, intranasal administration of vaccines presents advantages such as the activation of mucosal immunity, which is very relevant for respiratory pathogens, and a reduced reliance on needle-based immunizations. The MC3 LNP has been used to deliver a 4.5 kb nucleoside-modified sequence encoding the cystic fibrosis transmembrane conductance regulator (CFTR) to mice [127]. A luciferase reporter was successfully expressed in the lungs by pipetting a 12 μg dose into the nostrils for spontaneous inhalation. Then, in a transgenic CFTR knockout mouse, application of CFTR mRNA LNPs restored CFTR-mediated chloride secretion to conductive airway epithelia for at least 14 days. MC3 LNPs were used again in a subsequent study of delivery to the nasal epithelium by using a luciferase reporter. Here, the use of a nebulizer to create an aerosol using the LNPs was examined; however, aerosolization resulted in LNP aggregation, doubling their size to 170 nm and resulting in a loss of transfection activity in vitro [128]. As a result, the researchers decided to instill the LNPs into the nostrils and found the luciferase reporter mainly expressed in nasal epithelia, with some additional transfection in lung epithelia. This study highlighted the delivery challenges of obtaining uniform and high levels of mRNA transfection in nasal and lung epithelia. Intranasal delivery of mRNA LNPs was also achieved using the older DOTAP/cholesterol/PEG–lipid system combined with protamine to encapsulate non-modified mRNA-expressing cytokeratin 19 in order to provoke a cellular immune response and slow tumor growth in a Lewis lung cancer xenograft model in mice [129]. These LNPs were large, 170 nm in size, and cationic, with 10 mV zeta potential and the ability to transfect 30% of DC2.4 dendritic cells in vitro. Once the xenograft tumor was established, 10 μg of cytokeratin 14-encoding mRNA LNPs was intranasally instilled in 100 μL PBS once per week for 3 weeks, resulting in a very significant reduction in tumor volume growth compared to the PBS control. A nucleoside-modified mRNA encoding the influenza antigen H3N2-HA was delivered in another study using DOTAP/DOPE/PEG–lipid LNPs, as well as in the same LNP-bearing mannose as a ligand to facilitate uptake by macrophages and dendritic cells [130]. These LNPs were also large, at 200 nm, positively charged, at 15 mV zeta potential, and able to express luciferase in the lungs following intranasal instillation of a 12 μg dose. Two 12 μg doses of the H3N2-HA LNPs were instilled intranasally at weeks 0 and 3 in C57BL/6 mice that were subsequently challenged with a

lethal dose of H1N1. Both LNPs containing the mRNA-encoded immunogen were capable of complete protection, while the mannose-coated LNP appeared more able to also block weight loss. Intranasal administration of LNPs appears feasible, although the doses were higher than those reported for intramuscular administration and the method of installation or aerosolization still requires further development.

8. Delivery of mRNA LNPs Encoding Antibodies

More than 70 monoclonal antibodies (mAbs) are currently on the market, with global sales of 125 billion USD. The possibility of using mRNA-encoded antibodies may bring some advantages, including endogenous protein synthesis benefiting from native post-translation modifications and a simplified manufacturing method that does not require cell culture and extensive purification and characterization of the protein product [8]. The feasibility of delivering mRNA-encoded mAbs for passive immunization was shown by the encapsulation of purified nucleoside-modified mRNAs encoding the light and heavy chains of VRC01, a broadly neutralizing antibody against HIV-1, into Acuitas LNPs [131]. Balb/c mice receiving a 30 µg dose IV that would target hepatocytes expressed the mAbs for more than a week, with serum levels reaching 150 µg/mL, which was higher than that obtained by direct injection of 600 µg of the mAb, with weekly injections capable of maintaining a constant serum level above 40 µg/mL. Both a 30 µg and a 15 µg injection of the mRNA LNP could protect CD34-NSG humanized mice from an HIV-1 challenge given 24 h later, as indicated by analyses of serum for viral RNA copies 2 weeks post challenge. The feasibility of therapeutic non-modified mRNA-encoded antibodies was confirmed in a study by CureVac, also using Acuitas LNPs [25], where an IgG mAb with broad neutralization ability for a variety of rabies strains was chosen, as well as a heavy chain-only Vh domain-based (VHH) neutralizing agent against the botulinum toxin [132]. An mRNA-encoded rituximab, targeting CD20, the gold standard for treating non-Hodgkin's lymphoma, was also produced. The animal studies here used an Acuitas LNP that targeted hepatocytes by IV administration. A single administration of 40 µg in mice produced serum levels of IgG just above 10 µg/mL, which gradually declined to 1 µg/mL after 1 month. The same dose of the VHH single-domain neutralizing agent produced 10-fold higher levels, but with a much shorter half-life of several days due to the absence of the Fc region. Single IV administration of 40 µg in mice was also able to entirely protect mice when administered either 1 day before or 2 h after a lethal challenge of the rabies virus. Similarly, a 40 µg dose 6 h after a lethal botulinum toxin challenge entirely protected the animals. A third challenge model, where Raji-luc2 B-cell lymphoma cells were engrafted intravenously and allowed to grow for 4 days and then 10 or 50 µg of mRNA-encoded rituximab in the Acuitas LNP was administered five times over 18 days, resulted in all animals surviving this lethal tumor challenge and the 50 µg dose was able to entirely abrogate tumor growth.

Bispecific antibodies that recruit T cells to tumor cells were also encoded in modified mRNA constructs and delivered in vivo using a commercial transfection reagent, TransIT, which is not as efficient as current LNPs for liver delivery [133]. The mRNA construct could sustain circulating and bioactive bispecific antibodies for more than 6 days, while the same 5 µg dose of the protein-bispecific antibody was reduced to near baseline after one day. A second study was also carried out using bispecific antibodies in the VHH format, where one VHH that binds the conserved influenza A matrix protein 2 ectodomain (M2e) was genetically linked to a second VHH that specifically binds to the mouse Fcγ receptor IV (FcγRIV) in order to recruit innate immune cells expressing FcγRIV to influenza infected cells expressing M2e [134]. These nucleoside-modified mRNA constructs were delivered using DOTAP/cholesterol LNPs by intratracheal instillation into the mouse lung and, 4 h later, challenged with a lethal influenza virus dose. Most of the mice (80%) were protected from the lethal dose, although they did experience significant weight loss and the DOTAP/cholesterol mRNA nanoparticles resulted in a temporary influx of granulocytes in the lungs, combined with an increase in serum IL-6 cytokine levels. Finally, a potent

neutralizing antibody identified in the B cells of a survivor of chikungunya infection was encoded in a nucleoside-modified mRNA construct delivered in an LNP possibly containing MC3 or Lipid 5 [135]. Protection against viral challenge administrated 24 h pre-infusion in mice was achieved at 0.5 mg/kg (10 μg) IV for the mRNA-encoded mAb, while 2 mg/kg of the protein mAb was needed. Therapeutic protection by infusion at 4 h post infection was obtained at very high doses of 10 mg/kg (200 μg) in mice. Non-human primate studies found that very high doses up to 3 mg/kg (9 mg) produced minimal transient toxicity involving splenic enlargement and increased CCL2 serum levels, and the antibody was detectable for several months post infusion. Based on these results, Moderna initiated a phase 1 human trial and announced positive results where infusions of 0.1 and 0.3 mg/kg were well tolerated and resulted in serum levels of the mAb in the 1–14 μg/mL range that are expected to be protective against chikungunya virus for up to 16 weeks after a single dose [136].

9. Assembly and Structure of Lipid Nanoparticles

The current methods of mRNA lipid nanoparticle production utilize microfluidic or T-junction mixing to rapidly combine an ethanol phase containing the hydrophobic lipids and an aqueous phase that contains the mRNA in a buffer, such as acetic acid, at pH 4 (Figure 2). Prior methods, such as thin film hydration and ethanol injection, are generally not used since they result in heterogeneous larger-sized nanoparticles with lower mRNA encapsulation efficiency, which are difficult to scale up [95]. Microfluidic mixing has the advantage of being able to mix very small volumes of lipids in ethanol with mRNA in aqueous solutions (tens of μL) so that the screening of many components and formulation parameters is possible. T-mixing, on the other hand, is the general method of choice for the commercial production of large batches of mRNA LNPs, such as those in current clinical trials. A recent publication demonstrated that both methods result in LNPs of similar sizes and morphologies [96]. The rapid mixing of the two solutions is key in order to limit the resultant particle size to <100 nm, thus obviating the need for the size reduction methods (extrusion, sonication) required by other production methods [137]. The assembly and formation of the LNPs from these solutions is driven by both hydrophobic and electrostatic forces, as depicted in Figure 2. The four lipids (ionizable lipid, DSPC, cholesterol, PEG–lipid) are initially soluble in ethanol without any counterions present so that the ionizable lipid is unprotonated and electrically neutral (Figure 2A). One volume of the lipid-containing ethanol solution is typically mixed with three volumes of mRNA in a pH = 4 aqueous acetate buffer so that when the lipids contact the aqueous buffer they become insoluble in a 3:1 water/ethanol solvent and the ionizable lipid becomes protonated and positively charged, which then drives it to electrostatically bind to the negatively charged phosphate backbone of the mRNA (Figure 2B), while the lipids become insoluble, forming a lipid particle encapsulating the mRNA in a primarily aqueous suspension. A key component in this process is the PEG–lipid, since the PEG chain is hydrophilic and thereby coats the particle and also determines its final thermodynamically stable size. By changing the mole fraction of PEG, the LNP size can be predictably controlled, for example, from 100 nm at a 0.5% mole fraction to 43 nm at a 3% mole fraction of PEG–lipid [89]. A recent critically important observation was that LNP structure and size continue to evolve post-mixing when the mRNA LNP suspension is either diluted in aqueous buffer or dialyzed against an aqueous buffer to both raise the pH and eliminate ethanol [96]. The initial mixing of aqueous and lipid phases produces a pH near 5.5, protonating the ionizable lipid, which has an LNP pKa of near 6.5 and allows mRNA binding and encapsulation (Figure 2B,C). Subsequent raising of the pH by dilution, dialysis or tangential flow filtration neutralizes the ionizable lipid until it is mainly uncharged at pH 7.4 (Figure 2D). As the ionizable lipid becomes neutral, it also becomes less soluble, resulting in the formation of larger hydrophobic lipid domains that drive the fusion process of the LNPs so that their size increases and the core of the LNP becomes an amorphous electron-dense phase, mainly containing the ionizable lipid bound to the mRNA. It was estimated that as many as 36 vesicles could fuse

to form just one final LNP during this process (Figure 2C,D). The fusion was demonstrated using FRET pairs and the role of the PEG–lipid was further seen to occur during this process since adding the PEG–lipid after mixing controlled the final LNP size in the same way as adding the PEG–lipid before mixing [138]. This study and another study using neutron scattering methods have also shown that DSPC forms a bilayer just underneath the peripheral PEG layer in the LNP, whose central core is primarily the ionizable lipid bound to mRNA (Figure 2D). Cholesterol is thought to be distributed throughout the LNP [89].
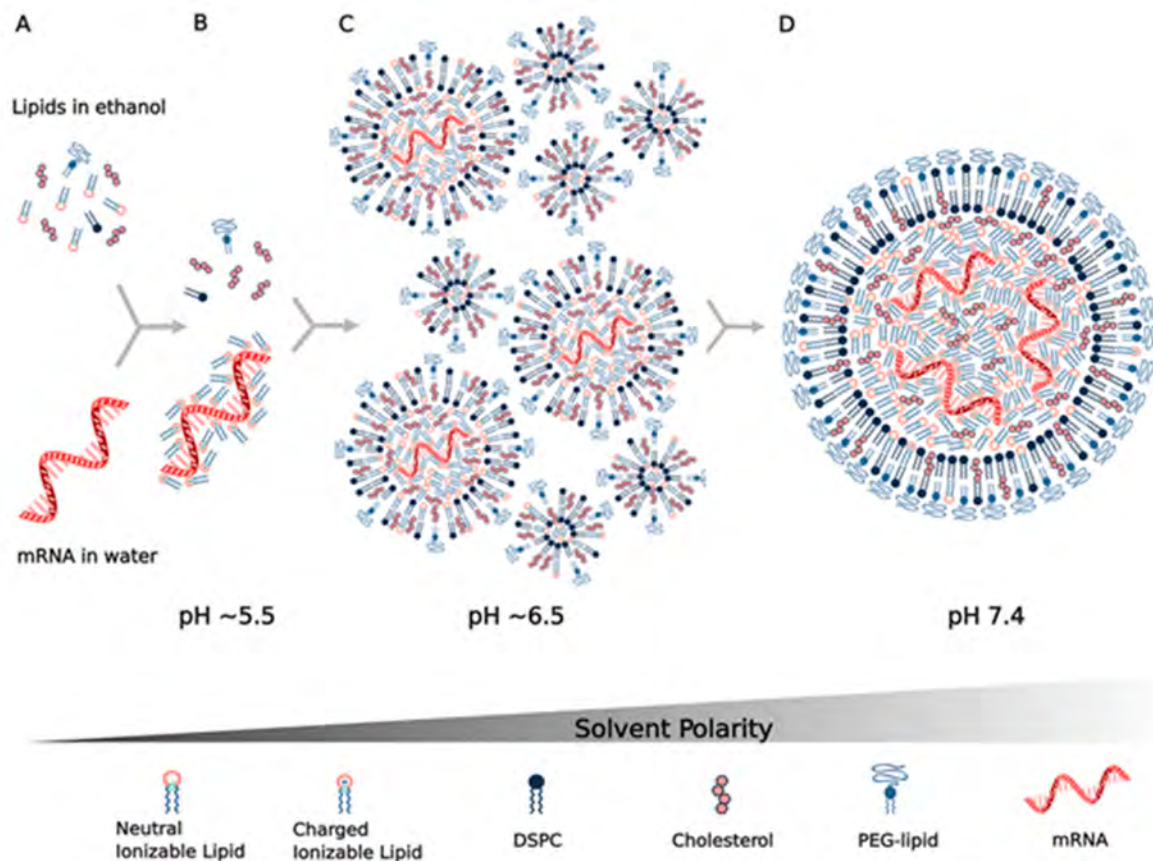
Figure 2. mRNA lipid nanoparticle assembly is achieved by (**A**) rapid mixing in a microfluidic or T-junction mixer of four lipids (ionizable lipid, DSPC, cholesterol, PEG–lipid) in ethanol with mRNA in an aqueous buffer near pH4. (**B**) When the ionizable lipid meets the aqueous phase, it becomes protonated at a pH ~5.5, which is intermediate between the pKa of the buffer and that of the ionizable lipid. (**C**) The ionizable lipid then electrostatically binds the anionic phosphate backbone of the mRNA while it experiences hydrophobicity in the aqueous phase, driving vesicle formation and mRNA encapsulation. (**D**) After initial vesicle formation, the pH is raised by dilution, dialysis or filtration, which results in the neutralization of the ionizable lipid, rendering it more hydrophobic and thereby driving vesicles to fuse and causing the further sequestration of the ionizable lipid with mRNA into the interior of the solid lipid nanoparticles. The PEG–lipid content stops the fusion process by providing the LNP with a hydrophilic exterior, determining its thermodynamically stable size, and the bilayer forming DSPC is present just underneath this PEG–lipid layer.

10. Determinants of Performance of mRNA Delivery Systems for Vaccines

The determinants of performance for mRNA delivery systems are multifactorial and interacting and include: (1) their potency or ability to deliver to the appropriate cell and efficiently release mRNA to the cytoplasmic translational machinery; (2) their adjuvanticity, which can boost the immune response; and (3) the minimization of any contribution to adverse events or toxicity that could arise from excessive inflammation at the injection site or systemic distribution and off-target expression.

10.1. Dose

The potency of mRNA delivery systems is most easily appreciated by the large range of doses that are currently being pursued in SARS-CoV-2 clinical trials, from 1 to 100 µg (Table 1). Doses in human trials are clearly grouped into the higher 30–100 µg doses for nucleoside-modified RNA (Moderna, BioNTech), lower 7.5–20 µg doses for unmodified RNA (CureVac, Translate Bio), and even lower 1–10 µg doses for self-amplifying RNA (Arcturus, Imperial College of London). Two factors are at play in determining these doses: the level of neutralizing antibody titers and T cell responses achieved versus convalescent plasma, and the frequency and severity of adverse events incurred at each dose. There appears to be a fairly narrow window of acceptance where the doses required to achieve protection are also close to generating an unacceptable frequency and severity of adverse events, as evidenced by the discontinuation of the highest doses tested in all phase 1 clinical trials. Both modified nucleoside constructs tested in the BioNTech phase 1 trials had high neutralizing titers versus convalescent plasma, while the larger construct encoding the membrane-bound full-length spike protein had a lower frequency and severity of adverse events, leading to its selection for the phase 3 study. Notably, dose is represented as mass, while the molar dose is dependent on the length of the construct and, furthermore, the amount of mRNA actually being translated is a small fraction of either, depending on the efficiency and targeting properties of the delivery system.

In animal studies of prophylactic mRNA vaccines for infectious diseases, the initial doses capable of producing neutralizing antibodies or protection against viral challenge were quite high in the 10–80 µg range for mice when using protamine, dendrimers and early cationic lipid systems (Table 3). When the more recent LNPs were subsequently used, the dose required for neutralization in mice was considerably reduced to near the 1 µg level when given twice, while for non-modified mRNA the dose appears to be lower, near 0.25 µg. The dose can be lower again for self-amplifying mRNA, such as 0.1 µg given twice or 2 µg given once. In larger animal models (hamster, ferret and non-human primate), fewer studies are available and the doses fall into a wide range of 5 µg to 200 µg with no apparent pattern. Interestingly, when using body surface area to convert human doses to animal doses, a 100 µg dose for a 60 kg human would be equivalent to a 15 µg dose in a 3 kg rhesus macaque and to a 0.4 µg dose in a 20 g mouse [139], numbers that approximate those of LNPs in Tables 1 and 3. The delivery system clearly plays an important role in determining the effective dose. There is a strong desire to improve delivery efficiency in order to reduce dose and maintain potency since this is expected to reduce adverse event frequency and severity by reducing the local reactions and off-target effects of the mRNA and of the delivery vehicle. Reducing the dose will also lower the amount of raw material needed and the cost associated with vaccinating each individual. In particular, the current COVID-19 pandemic has brought into focus some significant supply chain and production capacity limitations of mRNA LNP vaccines that could be improved with more efficient delivery systems.

Table 3. mRNA doses in in vivo prophylactic vaccination. The mRNA dose required to induce neutralizing antibody titers, or the dose that provides protection against viral challenge, is shown for different mRNA delivery systems and in different species. The advent of lipid nanoparticles (LNPs) for mRNA delivery reduced the required doses by ~10-fold compared to earlier delivery systems.

Delivery System	mRNA Type	Species	Dose	Readout	Reference
Naked mRNA	non-modified	mouse	80 µg twice	protection	[140]
Naked mRNA	self-amplifying	mouse	1.25 µg twice	protection	[140]
Protamine	non-modified	mouse	10 µg twice	neutralizing titers	[66]
Protamine	non-modified	mouse	80 µg twice	protection	[66]
Modified Dendrimer	self-amplifying	mouse	40 µg once or 4 µg twice	neutralizing titers	[126]
DOTAP/DOPE/PEG	nucleoside modified	mouse	12 µg twice intranasal	neutralizing titers and protection	[130]
Cationic Nanoemulsion	self-amplifying	mouse	15 µg twice	neutralizing titers	[70]
Nanostructured Lipid Carrier	self-amplifying	mouse	0.1 µg once	neutralizing titers	[71]
LNP (Acuitas)	non-modified	mouse	0.5 µg twice	neutralizing titer	[69]
LNP (MC3)	nucleoside-modified	mouse	10 µg once or 2 µg twice	protection	[99]
LNP (MC3)	nucleoside-modified	mouse	0.4 µg once	protection	[97]
LNP (Acuitas)	nucleoside-modified	mouse	0.5 µg once	protection	[141]
LNP (Acuitas)	nucleoside-modified	mouse	1 µg twice	neutralizing titers	[49]
LNP (Moderna)	nucleoside-modified	mouse	1 µg twice	neutralizing titers and protection	[45]
LNP (Acuitas)	non-modified	mouse	0.25 µg twice	neutralizing titers	[53]
LNP (Translate Bio)	non-modified	mouse	0.2 µg twice	neutralizing titers	[56]
LNP (Arcturus)	self-amplifying	mouse	2 µg once	neutralizing titers and protection	[58]
LNP (Acuitas)	self-amplifying	mouse	0.1 µg twice	neutralizing titers	[60]
LNP (Acuitas)	non-modified	Syrian Hamster	10 µg twice	protection	[53]
LNP (MC3)	nucleoside-modified	ferret	50 µg once	neutralizing titers	[97]
Cationic Nanoemulsion	self-amplifying	non-human primate	75 µg twice	neutralizing titers	[70]
LNP (MC3)	nucleoside-modified	non-human primate	200 µg twice	neutralizing titers	[97]
LNP (MC3 or Moderna Lipid H)	nucleoside-modified	non-human primate	5 µg twice	neutralizing titers	[42]
LNP (Acuitas)	nucleoside-modified	non-human primate	30 µg twice	neutralizing titers	[49]
LNP (Moderna)	nucleoside-modified	non-human primate	100 µg twice	neutralizing titers	[45]
LNP (Translate Bio)	non-modified	non-human primate	15 µg twice	neutralizing titers	[56]

10.2. Potency and Delivery Efficiency

There have been many studies that have attempted to identify structure–function relationships for LNP and other nucleic acid delivery systems. The most commonly cited feature of the LNP that determines its potency or delivery efficiency is its pKa. The pKa is the pH at which 50% of the ionizable lipid in the LNP is protonated. To date, the LNP pKa has only been measured with a dye-binding assay called TNS, which is negatively charged and experiences fluorescence enhancement upon binding a positively charged LNP [88]. Fluorescence measurement of LNPs incubated with TNS in buffers covering a wide range of pH values is used to deduce dye binding to surface charge and the pKa

estimated, where half of the maximal fluorescence is attained. It was well established that the MC3-based Onpattro LNP had an optimal pKa of 6.4 for silencing hepatocytes after IV administration [92]. There was a very sharp optimum in TNS pKa in the range of 6.2–6.8 for any LNP to effect hepatocyte silencing. An excellent model for explaining this pKa dependence was based on the ionizable lipid in the LNP being near neutral at pH 7.4 while, after internalization into a cell, the pH of the endosome will begin to drop as it evolves through the endolysosomal pathway, thereby progressively protonating the ionizable lipid, which will then bind to an anionic endogenous phospholipid of the endosome and disrupt its bilayer structure to release the mRNA into the cytoplasm for ribosomal loading [17]. Endosomal disruption requires an additional feature of the ionizable lipid, namely a cone-shaped morphology where the cross-section of the lipid tails is larger than that of its head group. This renders the ionizable lipid/endosomal phospholipid ion pair incompatible with a bilayer and more likely to form structures such as inverted hexagonal phases that can disrupt the endosomal membrane. This has been called the molecular shape hypothesis [91] and is the mechanism explaining why the introduction of one or two double bonds into a saturated C18 alkyl chain generates a more cone-shaped and less cylindrical morphology that is membrane disrupting and endosomolytic [88]. These two C18 linoleic acid tails, combined with an appropriately tuned pKa of the dimethylamine headgroup, are the defining features of the MC3 ionizable lipid. The ionizable lipids that have replaced MC3 for mRNA delivery conserve the pKa requirement, but pursue greater endosomolytic character by introducing more branching into the alkyl tails. Lipid H and Lipid 5 from Moderna, for example, have three alkyl tails, as does Lipid 2,2 (8,8) 4C CH3 from Arcturus, while Acuitas ALC-0315 has four and A9 has five alkyl tails (Table 2). This augmented cone-shaped morphology is presumably the reason why LNPs that incorporate these ionizable lipids are more efficient delivery vehicles with greater endosomal release.

Although LNP pKa and the molecular shape hypothesis are well established as contributing to LNP delivery efficiency, other factors are important as well, such as the stability of the PEG–lipid on the LNP surface, and the proportions of the four lipids in the ethanol solution, which ultimately determine the LNP ultrastructure. The PEG–lipid controls LNP size, as mentioned above, by providing a hydrophilic shell that limits vesicle fusion during assembly so that higher PEG–lipid concentrations produce smaller LNPs. For example, one study showed that varying the mole fraction of the PEG–lipid from 0.25% to 5% reduced the LNP size from 117 nm to 25 nm and that the optimal size for hepatocyte silencing was 78 nm, generated with 2.5% PEG–lipid [142]. Since the alkyl tail of the PEG–lipid had 14 carbons, it was not stably anchored to the LNP surface and was found to be gradually shed from the LNP in circulation, along with the shedding of the ionizable lipid MC3 and DSPC. This PEG shedding is thought to render the LNP transfection competent at some point, but, if too extreme, results in the rapid loss of the ionizable lipid and DSPC, which will negatively impact endosomal release. For example, by extending the alkyl tail to 18 carbons, the PEG–lipid did not shed, but was also not silenced in hepatocytes. On the other hand, adding higher concentrations of PEG to make smaller particles resulted in faster shedding, loss of the ionizable lipid and reduced silencing. The labile and dynamic nature of the LNP is currently only partly understood. Another study also found that an intermediate sized 64 nm diameter LNP made with 1.5% PEG–lipid was more efficient for mRNA delivery than a larger one at 100 nm (0.5% PEG–lipid), as well as a smaller LNP at 48 nm (3% PEG–lipid), similar to the study mentioned above [89]. However, by changing the mole ratios of the four lipids in order to conserve a calculated density of DSPC under the PEG layer of the LNP at the optimal value found in the 64 nm 1.5% PEG–lipid LNP, these authors were able to make larger 100 nm LNPs with a twofold increase in mRNA expression compared to the 64 nm-sized LNPs. Thus, in addition to the LNP pKa, ionizable lipid molecular shape and the dynamics of the PEG–lipid, more detailed features of the LNP ultrastructure and the state of each component are also important in determining potency.

10.3. Endosomal Release

Cell uptake and endosomal trafficking of siRNA-LNPs were studied in detail and are assumed to be similar to the uptake and endosomal trafficking of mRNA LNPs. With the MC3 LNP, a quantitative study using colloidal gold particle counting in electron microscopy showed that only 2% of siRNA that were in endosomes actually escaped from endosomes into the cytosol, resulting in a few thousand siRNA molecules per cell that were available for silencing [106]. This number was, however, in the same range as the estimated levels of functionally active siRNAs interacting with RISC per cell at therapeutically relevant concentrations. Thus, the vast majority of siRNA was destined for lysosomal degradation or recycling through multivesicular bodies (late endosomes) for release in the exosomes [143,144]. Increasing the endosomolytic behavior of LNPs is the central approach to improving delivery efficiency, mainly through pKa adjustment of the LNP and by increasing the cone-shaped morphology of the ionizable lipid. For the latter, Lipid H [42] and Lipid 5 [101], which contain three branches versus two in MC3, but with similar pKa, increased endosomal release fourfold compared to MC3. Endosomal release has not been reported for Acuitas ALC-0315; however, its hepatocyte silencing efficiency was 10-fold higher than MC3 [47], suggesting its more cone-shaped four-branch structure also had higher endosomal release. These newer generation ionizable lipids therefore appear to achieve a endosomal release, closer to 15% or higher compared to the 2–5% found for MC3 siRNA-LNPs. One of the challenges in this area is the lack of a reliable standardized endosomal release method that can be implemented broadly. Many methods have been developed, but are usually specific to only one lab group [42,101,145–149]. mRNA was also recently shown to undergo exocytosis in an amount that is similar to the amount released into the cytosol [150]. MC3 LNPs disassembled in late endosomes and NP 1 complexes of MC3 and the mRNA were repackaged into exosomes that were exported from the cell. These endo–exosomes maintained an mRNA delivery capacity that was similar to the original MC3 LNPs from which they were derived, but could traffic to different tissues and appeared to be less immune activating. The potential significance of this exosomal redistribution of mRNA delivered by LNPs remains to be explored.

10.4. Charge and Ligand Mediated Targeting

The early lipid nanoparticles using permanently charged cationic nonionizable lipids were large and, due to their permanent positive charge, were quickly opsonized and generally targeted the lung. The group at BioNTech reduced the amount of cationic DOTMA in DOTMA/DOPE mRNA LNPs until the net charge was negative due to an excess of anionic mRNA at NP ratios of less than one. Injecting these negatively charged and large 300 nm mRNA LNPs intravenously led to spleen targeting and mRNA expression in dendritic cells and they were able to mediate adaptive as well as type I IFN-mediated innate immune mechanisms for cancer immunotherapy [151]. Similarly, spleen-targeting mRNA LNPs were produced using the C12-200 prototype LNP, but replacing C12-200 with the small dendritic ionizable lipid Cf-Deg-Lin, which has four linoleic acid alkyl chains and four nitrogen atoms with a TNS pKa of 5.7. This very low pKa of the LNP would ensure that the ionizable lipid was not protonated until it reached a pH below 7, creating an LNP that would bear a net negative charge from the mRNA until quite late in the endosomal pathway and therefore similarly traffic to the spleen [152]. They found that the major cell population in the spleen to express the mRNA were B lymphocytes, where 7% of B lymphocytes were expressed the mRNA according to flow cytometric analyses. More recently, charge-mediated targeting was achieved using three different basic LNPs with MC3, C12-200, or 5A2-SC8 as ionizable lipids mixed in a certain mole fraction of a permanently cationic lipid (DOTAP) or a permanently anionic lipid (18PA) to endow the LNPs with a net positive, net negative or an intermediate near-neutral net charge [153]. Consistent with the above findings, highly positive LNPs targeted the lungs and highly negative LNPs targeted the spleen, while intermediate charge levels predominantly targeted the liver. Liver targeting

has been shown to depend on Apo-E binding to near-neutral liposomes or LNPs [154], which does not occur for negatively charged liposomes [155].

Notably, all of the above charge-mediated targeting studies have been done using IV administration and the routes typically used for vaccination, such as the intramuscular or intradermal routes, have not been examined. Most studies that analyze expression after intramuscular injection do, however, detect the systemic trafficking of mRNA LNPs, which are rapidly and strongly expressed in the liver, at the same time as they are expressed in muscle and draining lymph nodes [97,156,157]. These particular LNPs therefore seem to enter the vasculature and are subsequently expressed in liver hepatocytes due to passive ApoE-mediated targeting, which is not surprising since they were designed for hepatocyte targeting. This systemic distribution and expression of immunogens could, however, generate systemic cytokines, complement activation and lead to other potential undesirable effects that could amplify the frequency or severity of adverse events and/or impair immune response generation. Finally, only a limited number of studies have been carried out with ligand-mediated targeting of LNPs. Lung endothelial cell targeting was achieved by conjugating CD31 (PECAM) antibodies to the LNP and injecting intravascularly [158]. The liver hepatocyte-directed LNP then became largely redirected to the lung. A similar approach using a VCAM ligand successfully targeted LNPs to inflamed regions of the brain and alleviated TNF-α-induced brain edema [159]. Dendritic cells in vitro were also more efficiently transfected using a mannosylated liposome, which may be a strategy applicable to vaccination [160]. Higher throughput screening methods to identify ligands targeting specific cell types have also been developed and may be applicable for the targeting of specific dendritic cell subsets [161,162].

10.5. Adjuvanticity of the Lipid Nanoparticle

The lipid nanoparticle is known to have its own adjuvant activity. A study in mice at a 10 µg dose and nonhuman primates at a 100 µg dose of nucleoside-modified mRNA LNPs (from Acuitas) encoding various immunogens showed increased numbers of antigen-specific T follicular helper (Tfh) cells and germinal center B (GC B) cells compared to an inactivated virus [33]. Tfh cells drive immunoglobulin class switch, affinity maturation, and long-term B cell memory and plasma cells. An adjuvant property of the LNP itself was found when an FLuc mRNA LNP was co-administered with a protein subunit HA immunogen and increased germinal center B cell numbers fourfold, although the number of Tfh cells was not increased compared to the protein alone. The LNP thus appears to be amplifying GC B cell responses, in particular to a nucleoside-modified mRNA LNP. Another study using an asymmetric ionizable lipid from Merck investigated the use of LNPs as adjuvants for Hepatitis B protein subunit vaccines [163]. Co-administering LNPs with the protein subunit vaccine enhanced B cell responses to levels comparable to known vaccine adjuvants, including aluminum-based adjuvant, an oligonucleotide and a TLR4 agonist, 3-O-deactytaledmonophosphoryl lipid A (MPL). The LNPs elicited potent antigen-specific CD4+ and CD8+ T cell responses and the Th1 vs. Th2 bias could be further influenced by the inclusion of additional adjuvants within the LNP. A follow-up study by this group using a Dengue virus immunogen found a similarly strong adjuvant activity in the LNP and that this activity depended on the presence of the ionizable lipid [164]. The lipid components in liposomes have also been previously recognized as having adjuvant activity in mucosal vaccines [165,166].

10.6. Injection Site Reactions, Safety, Tolerability, Reactogenicity of mRNA LNPs

A general safety study for MC3 nucleoside-modified mRNA LNPs expressing hEPO via IV administration to liver in rats and non-human primates found mild toxicological events up to 0.3 mg/kg, which is more than 10-fold the expected therapeutic dose [167]. The main findings in the rats were increased white blood cell counts, changes in the coagulation parameters at all doses, as well as liver injury. Non-human primates showed lymphocyte depletion accompanied by mild and reversible complement activation. These results

were in line with an earlier toxicological study of the same LNPs for siRNA delivery [168], where rat mortality was noted at 6 mg/kg, while the no observable adverse effect level (NOAEL) was determined to be 1 mg/kg. Above 3 mg/kg elevations to serum chemistry parameters (ALT, AST, and TBIL), hematuria, and microscopic findings in the liver (vacuolation, inflammatory cell infiltrate, fibrosis, hemorrhage, and hepatocellular necrosis), spleen (lymphoid atrophy and necrosis) and kidney (tubular degeneration/regeneration) were noted. Safety findings in patients included infusion-related reactions (15% of patients, presumably complement mediated) and transient elevations of pro-inflammatory cytokines. Notably, the above doses administered IV, such as 0.3 mg/kg, are more than 10-fold higher than those in the current SARS-CoV-2 clinical trials that use IM administration. Nonetheless, these lower doses in the current human trials still induce a high frequency and sometimes moderate severity of both local injection site reactions and systemic adverse events. Currently, there is a paucity of published animal studies regarding correlates of these human adverse events in animals.

An extensive rhesus macaque study looking at the injection sites and trafficking of mRNA expression was performed using the MC3 LNP, delivering a nucleoside-modified mRNA encoding the influenza immunogen H10 mRNA intramuscularly or intradermally at a 50 μg dose [98]. They found a rapid cell infiltrate to the injection site within 4–24 h that could be driven by the LNP alone and was mainly composed of neutrophils and monocytes. The main cell types expressing mRNA were multiple monocyte and dendritic cell subsets at the injection sites and in the draining lymph nodes. Priming of T cell responses was restricted to the draining lymph nodes and the LNP alone did not induce CD80 in antigen-presenting cells. Ongoing generation of vaccine-specific CD4+ T cells occurred only in the vaccine-draining lymph nodes, where detection of mRNA-encoded antigens peaked at 24 h, whereas the antibody responses were sustained for weeks. Results consistent with the above were also reported using a non-modified mRNA encoding rabies virus glycoprotein G (RABV-G), delivered in an Acuitas LNP to mice with 0.5–10 μg doses and to non-human primates at 10 μg and 100 μg doses [69]. They also found that the LNP alone mediated cytokine generation in the muscle injection site and draining lymph nodes, but recognized that systemic detection of IL6 could occur due to trafficking through the blood and expression in the liver. Injection site erythema and edema were noted in the non-human primates at both 10 μg and 100 μg doses. It is also interesting to note that the LNPs used in mRNA delivery systems have a size with the range of 10–100 nm, which is known to be optimal for uptake into lymphatics, and that pegylation of lipids improves retention in lymphatics [169] and can reduce complement activation [109]. Since the emergency use approval of the Pfizer/BioNTech vaccine, there has been several observed incidences of acute anaphylaxis corresponding to 1 case in 100,000 vaccinations, which is about 10-fold the rate seen with other vaccines [170]. One possible source of this anaphylaxis is the prevalence of anti-PEG antibodies in the general population, which could trigger anaphylaxis in a patient subset due to the use of the PEG–lipid in LNPs. PEG-mediated anaphylaxis has been noted, for example, in a clinical contrast agent [171] and in a liposomal formulation of doxorubicin [172]. Nonetheless, the doses administered for the current SARS-CoV-2 vaccines correspond to a total PEG dose that is at least 15-fold lower than that found in those products, which seems to diminish this possibility. Another possibility is that the reactions are anaphylactoid in nature, but are non-specific responses to inflammation and other factors. A clinical study is underway to further elucidate this issue [173].

11. Conclusions

The progress of mRNA therapeutics has been extraordinary over the past two decades, beginning with the identification of means to control mRNA innate immunogenicity using modified nucleosides and sequence engineering, and the application of mRNA in vaccines and other therapeutic indications. The adoption of the lipid nanoparticle prototype from that used in siRNA delivery led to an order of magnitude improvement in delivery efficiency compared to previous systems and is continually improving, mainly due to the

design of new classes of ionizable lipids. Many aspects of mRNA LNP structure, function, potency, targeting and biological features, such as adjuvanticity, remain to be explored in order to fully exploit the potential of this powerful and transformative therapeutic modality.

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The Science and Practice of Pharmacy

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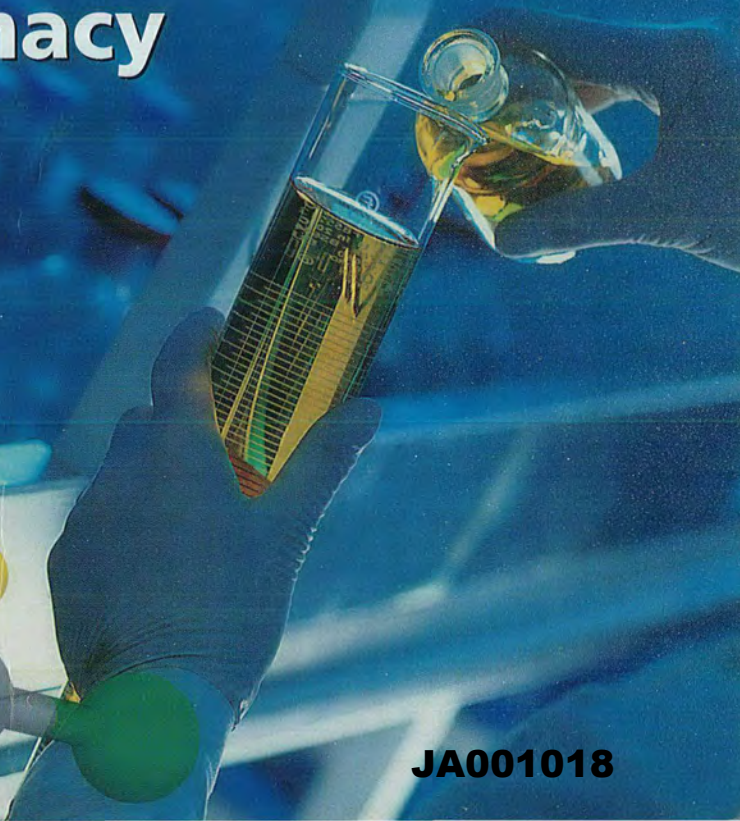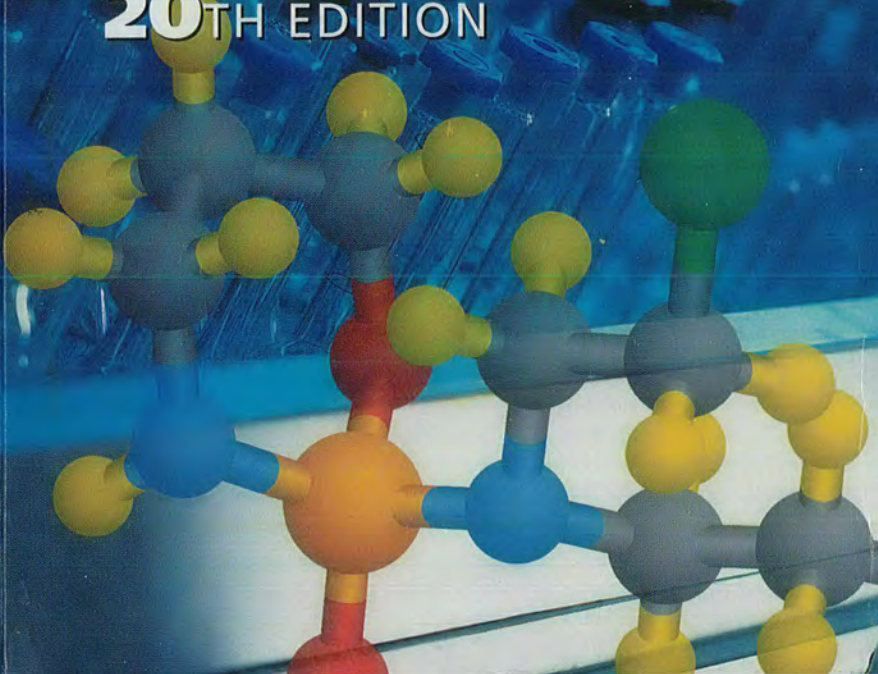
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Preface to the Twentieth Edition

At this writing, Remington, as it is commonly known in the profession, is at a point midway through the second decade of its second century. One hundred and fifteen years of service to students and practitioners, Remington is now poised on the brink of a new millenium. The 5 years that have elapsed since the publication of the previous edition have witnessed substantial and far-reaching changes in practically every field of human endeavor. Pharmacy—both the science and practice—perhaps has enjoyed more than its share. It is not so peculiar that a comment quite similar to the previous sentence has been incorporated in the first paragraph of practically every Preface of the previous 19 editions, including the first.

The impact of current information-exchange technology, with enormous quantities of data available at the touch of a key, engendered much consideration in the planning stages of this publication. At one time, a book of 2000 pages could be created using an all-inclusive approach: a tome with rather complete coverage of a field, in which one could find a wealth of information on practically every relevant subject. Without question, this approach is now beyond consideration. It was obvious that Remington 20 must address the newer concepts of information exchange. With modern technology, current information is processed and updated so rapidly that it may quickly become stale and, sometimes, of questionable value.

A wholesale transformation of the book was not envisioned, but rather a judicious and planned metamorphosis. Thus, a number of areas have been digested, reorganized, and pruned or amplified. Monographs for drugs have been culled, and most of the dosing and dosage form information has been deleted from individual drug monographs. This type of changeable information is impossible to keep current considering a 5-year publication cycle and the availability of the Internet. The general statements preceding individual classes of drugs have been expanded to provide comprehensive coverage of each class. Thus, useful information for the drug specialist is presented in an expanded format, rather than offered as overwhelming masses of data, which, if simply learned by rote, may easily be confused or forgotten.

The listing of names of manufacturers for each drug (official and/or generic) has been dropped. With the publication of the previous edition, it was evident that due to the dynamic situation in the drug industry, almost 25% of the pharmaceutical houses either had changed names or merged during the interval between completion of the manuscript and the publication date. Thus, this information often was either of little value or misleading.

One chapter (Calculus) has been deleted, and two chapters (Immunizing Agents and Diagnostic Skin Antigens [81] and Allergenic Extracts [82]) were consolidated into a single chapter [89] bearing the combined titles. Many chapters were trimmed in order to recover space for 10 new chapters, all within the realm of Pharmacy Practice. Several areas (such as Alternative Medicines and Treatment), which have enjoyed a resurgence of activity, have been expanded.

Essentially, the goal has been to reduce the clutter of excessive, easily attainable reference material and elaborate the area of pharmacy practice, without sacrificing the scientific concepts. The intention was to focus on the textbook features of the publication. This is a departure from the traditional role of a complete reference medium, to one that emphasizes teaching and learning principles while still retaining essential source material.

A number of previous authors have gone on to other interests and have transferred their tasks to new members of the team. This has exposed many areas to fresh ideas and alternative perspectives. The 143 authors/editors, of which 52 are first-time contributors, represent 34 universities, 17 pharmaceutical firms, 16 private practices, and 3 associated government agencies. Thus, practically every facet of the profession is encompassed.

Several members of the Editorial Board, who have served for a number of editions—Drs Hussar, Rippie, and Zink—have decided to relinquish their responsibilities. Their extensive contribution of time and effort is duly appreciated. A new member, Dr Nicholas Popovich, assumed the onus of a greatly expanded Part 8, Pharmacy Practice, and has completed the task superbly.

Mr John Hoover, associated with Remington for eight editions and currently as Managing Editor, has again done an exemplary job of coordination, especially at the early stages of manuscript preparation when all seems a clutter and disarray of paper (even in the "paperless" computer age!). Ms Bonnie Packer, our inveterate reader, critic, and editorial assistant, has untangled numerous word snarls, which are usually the product of overzealous authors and editors who have become immersed in their own disciplines and overlooked the fact that their words will also be read, and hopefully understood, by the novice.

It has been said that everything comes full circle or, in modern parlance, "What goes around comes around." In 1885, with the first edition of Remington, the publisher of this book was the well-established Philadelphia firm of JB Lippincott Co. This arrangement survived through eight editions, ending in 1936. With the intervention of the Second World War, the ninth edition did not appear until 1948 under the banner of the Mack Publishing Co, and this relationship has been sustained through the 19th edition in 1995. Mack has foregone the publishing role but will continue to print the book. The new publisher is, in reality, not so new, as the book is now in the hands of Lippincott Williams & Wilkins—the ring has been closed.

A volume of 2000 pages requires the cooperation of authors, editors, and the publisher and their associates in order to complete a tedious and time-consuming task. All who have contributed are to be commended for their efforts and time. However, having read the text at least twice in its entirety, the responsibility for errors, of any kind, ultimately resides with the editor. One can only work and strive to ensure that the book is free of flaws, blunders, and errors, but if this were the case, it probably would be a first.

Philadelphia, January 2000
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Preface to the First Edition

The rapid and substantial progress made in Pharmacy within the last decade has created a necessity for a work treating of the improved apparatus, the revised processes, and the recently introduced preparations of the age.

The vast advances made in theoretical and applied chemistry and physics have much to do with the development of pharmaceutical science, and these have been reflected in all the revised editions of the Pharmacopoeias which have been recently published.When the author was elected in 1874 to the chair of Theory and Practice of Pharmacy in the Philadelphia College of Pharmacy, the outlines of study which had been so carefully prepared for the classes by his eminent predecessors, Professor William Proctor, Jr, and Professor Edward Parrish, were found to be not strictly in accord, either in their arrangement of the subjects or in their method of treatment.Desiring to preserve the distinctive characteristics of each, an effort was at once made to frame a system which should embody their valuable features, embrace new subjects, and still retain that harmony of plan and proper sequence which are absolutely essential to the success of any system.

The strictly alphabetical classification of subjects which is now universally adopted by pharmacopoeias and dispensatories, although admirable in works of reference, presents an effectual stumbling block to the acquisition of pharmaceutical knowledge through systematic study; the vast accumulation of facts collected under each head arranged lexically, they necessarily have no connection with one another, and thus the saving of labor effected by considering similar groups together, and the value of the association of kindred subjects, are lost to the student.In the method of grouping the subjects which is herein adopted, the constant aim has been to arrange the latter in such a manner that the reader shall be gradually led from the consideration of elementary subjects to those which involve more advanced knowledge, whilst the groups themselves are so placed as to follow one another in a natural sequence.

The work is divided into six parts.Part I is devoted to detailed descriptions of apparatus and definitions and comments on general pharmaceutical processes.

The Official Preparations alone are considered in Part II. Due weight and prominence are thus given to the Pharmacopoeia, the National authority, which is now so thoroughly recognized.

In order to suit the convenience of pharmacists who prefer to *weigh solids* and *measure liquids,* the official formulas are expressed, in addition to parts by weight, in *avoirdupois weight* and *apothecaries' measure.*These equivalents are printed in *bold type* near the margin, and arranged so as to fit them for quick and accurate reference.

Part III treats of Inorganic Chemical Substances.Precedence is of course given to official preparation in these.The descriptions, solubilities, and tests for identity and impurities of each substance are systematically tabulated under its proper title.It is confidently believed that by this method of arrangement the valuable descriptive features of the Pharmacopoeia will be more prominently developed, ready reference facilitated, and close study of the details rendered easy. Each chemical operation is accompanied by equations, whilst the reaction is, in addition, explained in words.

The Carbon Compounds, or Organic Chemical Substances, are considered in Part IV.These are naturally grouped according to the physical and medical properties of their principal constituents, beginning with simple bodies like cellulin, gum, etc, and progressing to the most highly organized alkaloids, etc.

Part V is devoted to Extemporaneous Pharmacy.Care has been taken to treat of the practice which would be best adapted for the needs of the many pharmacists who conduct operations upon a moderate scale, rather than for those of the few who manage very large establishments.In this, as well as in other parts of the work, operations are illustrated which are conducted by manufacturing pharmacists.

Part VI contains a formulary of Pharmaceutical Preparations which have not been recognized by the Pharmacopoeia.The recipes selected are chiefly those which have been heretofore rather difficult of access to most pharmacists, yet such as are likely to be in request.Many private formulas are embraced in the collection; and such of the preparations of the old Pharmacopoeias as have not been included in the new edition, but are still in use, have been inserted.

In conclusion, the author ventures to express the hope that the work will prove an efficient help to the pharmaceutical student as well as to the pharmacist and the physician. Although the labor has been mainly performed amidst the harassing cares of active professional duties, and perfection is known to be unattainable, no pains have been spared to discover and correct errors and omissions in the text.The author's warmest acknowledgments, are tendered to Mr A B Taylor, Mr Joseph McCreery, and Mr George M Smith for their valuable assistance in revising the proof sheets, and to the latter especially for his work on the index.The outline illustrations, by Mr John Collins, were drawn either from the actual objects or from photographs taken by the author.

Philadelphia, October, 1885 JPR.

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CHAPTER **11**

Pharmaceutical Calculations

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The first technical operation that the student of pharmacy must learn is the manipulation of balances, weights, and measures of volume. This entails a study of the various systems of weights and measures, their relationships, and a mastery of the mathematics involved. This chapter considers the fundamental principles of metrology underlying the testing, manufacturing, and compounding of pharmaceutical preparations:

> **Weights and Measures**—An accumulation of facts concerning the various systems, with tables of conversion factors and practical equivalents. The relationships among the various systems of weights and measures are clarified.
> **Weighing and Measuring**—A discussion of the various types of balances, particularly prescription balances and methods of using, testing, and protecting them; also of various devices and methods of their use for measuring large or small volumes of fluids.
> **Density and Specific Gravity**—A consideration of the mass/volume ratio of a substance (density), and the ratio of the weight (mass) of one substance to the weight (mass) of another substance taken as the standard (specific gravity).

WEIGHTS AND MEASURES

Weight is a measure of the gravitational force acting on a body; weight is directly proportional to the body's mass. The latter, being a constant based on inertia, never varies, whereas weight varies slightly with latitude, altitude, temperature, and pressure. The effect of these factors usually is not considered unless very precise weighings and large quantities are involved.

Measure is the determination of the volume or extent of a body. Temperature and pressure have a pronounced effect, especially on gases or liquids. These factors, therefore, are considered when making precise measurements.

All standard weights and measures in the US are derived from or based on the United States National Prototype Standards of the Meter and the Kilogram. The standards are made of platinum-iridium, and are in the custody of the National Institute of Standards and Technology (NIST) in Washington, DC.

History

A brief outline of the origin of the many systems of weights and measures may help clarify the essential distinctions between them. The sense of the weight of a body cannot be conveyed intelligibly to the mind unless a means of comparison is chosen. As weight is the measure of the gravitational force of a body, this force is expressed in terms of standards of resistance, which exactly balance the body and keep it in equilibrium when used with a mechanical device constructed for this specific purpose. Such standards are termed *weights* and the mechanical devices are called *balances* or *scales*.

The standards that have been chosen by various nations are arbitrary, and instances are common where different standards are in use at the same time in the same country. Many of the ancient standards clearly are referable to variable parts of the human body, such as nail, foot, span, pace, cubit (length of the forearm), and fathom or faethm (stretch of the arms). In the history of metrology three periods may be traced:

1. The *Ancient* period, during which the old classical standards originated, terminated with the decline of the Roman Empire. The unit of distance used by all nations for maritime measurements, the *nautical* or *meridian* mile (1/60 of a degree of the earth's equatorial circumference) is exactly equal to 1000 Egyptian fathoms or 4000 Egyptian cubits. These Egyptian measurements, which have persisted for more than 4000 years, were based on astronomical or meridian measurements that were recorded imperishably in the great Pyramid at Ghizeh, whose perimeter is exactly 500 of these fathoms, or ½ nautical mile.
2. The *Medieval* period extended to the 16th century. During this period the old standards were lost, but their names were preserved, and European nations adopted various independent standards.
3. The *Modern* period extends from the 16th century to the present. Since the 17th century, the efforts of most enlightened nations have been directed toward scientific accuracy and simplicity, and during the present century toward international uniformity.

Historical metrology, also referred to as *documentary metrology*, is concerned with the study of monuments and records of ancient periods. *Inductive metrology* is concerned with the accumulation of data concerning the measurement of large numbers of objects that have been referred to as standards but which have no exact measure except by statutory regulation.

THE ENGLISH SYSTEMS—In Great Britain, in 1266, the 51st Act of the reign of Henry III declared

> "that by the consent of the whole realm of England the measure of the King was made—that is to say, that an English silver penny called the sterling, round and without clipping, shall weigh *thirty-two grains of wheat*, well dried and gathered out of the middle of the ear; and twenty pence (pennyweights) do make an ounce and twelve ounces a pound, and eight pounds do make a gallon of wine, and eight wine gallons do make a bushel, which is the eighth of a quarter."

The 16-ounce pound (*avoirdupois pound*), undoubtedly of Roman origin, was introduced at the time of the first civilization of the British island. However, according to Gray, the word

haberdepois was first used in English laws in 1303. A statute of Edward I (1304 AD) states "that every *pound* of money or of *medicines* is of *twenty shillings weight*, but the pound of all other things is *twenty-five shillings weight*. The *ounce of medicines* consists of *twenty pence*, and the *pound* contains *twelve* ounces [the Troy Pound], but in other things the pound contains *fifteen* ounces, in both cases the ounce weighing twenty pence."

These laws unfold the theory of the ancient weights and measures of Great Britain, and reveal the standards, ie, a natural object, grains of wheat. A difference existed then between the Troy and the avoirdupois pound, but the weights now in use are 1/16 heavier than those of Edward I, due to the change subsequently made in the value of the coin by the sovereign. In addition, the true pennyweight standard was lost, and, in the next revision of the weights and measures, the present troy and avoirdupois standards were adopted.

The *troy weight* is of still earlier origin. The great fairs of the 8th and 9th centuries were held at several French cities, including Troyes, the gathering place of traders from all countries. Coins frequently were mutilated, so they were sold by weight, and the standard weight of Troyes for selling coin was adopted for precious metals and medicines in all parts of Europe. The troy ounce and the avoirdupois ounce originally were intended to have the same weight, but after the revision it was found that the avoirdupois ounce was lighter by 42½ gr (grains) than the troy ounce. The subsequent adoption of troy weight by the London College of Physicians in 1618, on the recommendation of Sir Theodore Turquet de la Mayerne who compiled their first pharmacopoeia, has entailed upon all apothecaries who are governed by British customs to this day the very great inconvenience of buying and selling medicines by one system of weights (the *avoirdupois*) and compounding them by another (the *apothecary* or *troy*).

In the next century efforts were made toward reforming the standards, and in 1736 the Royal Society began the work that ended in the preparation, by Mr Bird under the direction of the House of Commons, of the standard *yard* and standard *pound* troy in 1760. Copies of these were prepared and no intentional deviation has been made since.

The growing popularity of the French metric system—and the desirability of securing a standard that could be recovered easily in case of loss or destruction, and that should be commensurable with a simple unit—prompted steps in England to secure these advantages in 1816. The labors of English scientists led to the adoption of the *imperial* measures and standards, which were legalized January 1, 1826; imperial standards are now in use in Great Britain, thus introducing another element of confusion into an already complicated subject. In this system the *yard* is equivalent to 36 inches, and its length was determined by comparison with a pendulum beating seconds of mean time, in a vacuum, at the temperature of 62°F at the level of the sea in the latitude of London, a length that was found to be 39.1393 inches. The *pound troy* (containing 5760 gr) was determined by comparison with a given measure of distilled water under specified conditions. Thus, a cubic inch of distilled water was weighed with brass weights in air at 62°F, the barometer at 30 inches, and it weighed 252.458 gr. The standard for measures of capacity in Great Britain (either dry or liquid) is the *imperial gallon*, which contains 10 lb avoir (each 7000 gr) of distilled water weighed in air at 62°F, the barometer standing at 30 inches. The *bushel* contains 8 such gallons.

Washington, in his first annual message to Congress, January 1790, recommended the establishment of uniformity in currency, weights, and measures. Action was taken with reference to the currency, and recommendations were made by Jefferson, then the Secretary of State, for the adoption of either the currently used English systems or a decimal system. However, nothing was accomplished until 1819 to 1820, when efforts again were made in the US to secure uniformity in the standards that were in use by the several states. Finally, after a lengthy investigation, on June 14, 1836, the Secretary of the Treasury was directed by Congress to furnish each state in the Union with a complete set of the revised standards, and thus the *troy pound* (5760 gr), the *avoirdupois pound* (7000 gr), and the *yard* (36 inches) are all identical with the British standards. However, the US *gallon* is quite different; the old wine gallon of 231 cu inches—containing 58,372.2 gr of distilled water at its maximum density, weighed in air at 62°F, the barometer standing at 30 inches—was retained. The bushel contained 77.274 lb of water under the same conditions, thus making the dry quart about 16% greater in volume than the liquid quart.

In 1864 the use of the metric measures was legalized in Great Britain, but was not made compulsory, and in 1866 the US followed the same course. By the US law of July 28, 1866, all lengths, areas, and cubic measures are derived from the international meter equivalent to 39.37 inches. Since 1893 the US Office of Standard Weights and Measures has been authorized to derive the yard from the meter: 1 yard equals 3600/3937 m, and the customary weights are referred to the kilogram by an Executive order approved April 5, 1893. Capacities were to be based on the equivalent; 1 dm^3 equals 1 liter, the decimeter being equal to 3.937 inches. The gallon still remains at 231 $inch^3$ and the bushel contains 2150.42 $inch^3$. This makes the liquid quart equal to 0.946 liter and the dry quart equal to 1.1013 liter, whereas the imperial quart is 1.1359 liter. The customary weights are derived from the international kilogram, based on the value that 1 avoir lb equals 453.5924277 g and that 5760/7000 avoir lb equals 1 troy lb.

Avoirdupois weight is used in general in the US for commercial purposes, including the buying and selling of drugs on a large scale and occasionally on prescription orders.

THE METRIC SYSTEM—The idea of adopting a scientific standard for the basis of metrology that could be reverified accurately was suggested by a number of individuals after the Renaissance. Jean Picard, the 17th-century French astronomer, proposed to take as a unit the length of a pendulum beating 1 sec of time at sea level, at latitude 45°.

In 1783, the English inventor James Watt first suggested the application of decimal notation, and the commensurability of weight, length, and volume. The French National Assembly in 1790 appointed a committee to decide the preferability of the pendulum standard or a terrestrial measure of some kind as a basis for the new system. The committee reported in 1791 in favor of the latter, and commissions were appointed to measure an arc of meridian and to perfect the details of the commensurability of the units and of nomenclature. However, certain inaccuracies were inherent in the early standards, so they do not bear to each other the intended exact relationships. The present accepted standards are defined in publications of the NIST.

In its original conception the meter was the fundamental unit of the metric system, and all units of length and capacity were to be derived directly from the meter, which was intended to be equal to one ten-millionth of the earth's quadrant. Furthermore, it originally was planned that the unit of mass, the kilogram, should be identical with the mass of a cubic decimeter of water at its maximum density. At present, however, the units of length and mass are defined independently of these conceptions.

For all practical purposes calibration of length standards in industry and scientific laboratories is accomplished by comparison with the material standard of length: the distance between two engraved lines on a platinum-iridium bar, the international prototype meter, which is kept at the International Bureau of Weights and Measures.

The *kilogram* is defined independently as the mass of a definite platinum-iridium standard, the *International Prototype Kilogram*, which also is kept at the International Bureau of Weights and Measures. The *liter* is defined as the volume of a kilogram of water, at standard atmospheric pressure, and at the temperature of its maximum density, approximately 4°C. The *meter* is thus the fundamental unit on which are based all metric standards and measurements of length and area and of volumes derived from linear measurements.

Of basic scientific interest is that on October 14, 1960, the 11th General Conference on Weights and Measures, meeting in Paris,

adopted a new international definition for the standard of length: the meter is now defined as the length equal to 1,650,763.73 wavelengths of the orange-red light of the krypton-86 isotope. This standard will be used in actual measurements only when extreme accuracy is needed.

The kilogram is the fundamental unit on which are based all metric standards of mass. The liter is a secondary or derived unit of capacity or volume. The liter is larger by about 27 ppm (parts per million) than the cube of the tenth of the meter (the cubic decimeter): 1 liter = 1.000027 dm³.

The conversion tables in this publication that involve the relative length of the yard and meter are based upon the relation: 1 m = 39.37 inch, contained in the act of Congress of 1866. From this relation it follows that 1 inch = 25.40005 mm (nearly).

In recent years engineering and industrial interests the world over have urged the adoption of the simpler relation, 1 inch = 25.4 mm exactly, which differs from the preceding value by only 2 ppm. This simpler relation has not as yet been adopted officially by either Great Britain or the US but is in wide industrial use.

In the US, the abbreviation *cc* (for cubic centimeter) still persists in general use and is taken as synonymous for the more correct milliliter. The US Pharmacopeia (USP) IX and National Formulary (NF) IV adopted the term *milliliter* with its abbreviated form *mil*, but it proved so unpopular in practice that the following pharmacopeial convention directed the return to the older term cubic centimeter (cc). However, in 1955, USP XV and NF X once again adopted the term milliliter with the abbreviation mL.

National jealousies and the natural antipathy to changing established customs interfered greatly with the adoption of the metric system during the early part of the 19th century. At present the metric system is in use in every major country of the world. In the US and Great Britain it is legalized for reference to and definition of other standards, and it is in exclusive use by nearly all scientists and by increasing segments of industry and the public. In the US the metric system was legalized in 1866, but not made compulsory; in the same year the international prototype meter and kilogram were adopted as fundamental standards. The US silver coinage was based upon the metric system, the half dollar being exactly 12 ½ g and the quarter and the dime being of the proportionate weights.

As corporations become more international, the need for a universal standard increased. Since 1875 there has been established and maintained an International Bureau of Weights and Measures, with headquarters at Paris. This Bureau is managed by an international committee that enjoys universal representation. One object of the committee is to make and provide prototypes of the meter and kilogram for the subscribing nations; approximately 40 such copies have been prepared.

The US prototype standards of both the meter and the kilogram mass, constructed of a platinum-iridium alloy, were brought from Paris in 1890 and are now in the custody of the National Institute of Standards and Technology (NIST) in Washington, DC. They have been reproduced and distributed by our own government to the various states having bureaus needing such replicas. The original US prototype meter was taken back to Paris in 1957 for reverification and was found to have altered only 3 parts in 100,000,000 after 67 years of use. Thus, there was no demonstrable change within the limits of experimental error.

Adoption of the krypton-86 wavelength of light definition for the meter gives the different countries the means to check their prototype meter bars without returning them to Paris at periodic intervals for comparison with the international meter bar.

Orthography and Reading

ORTHOGRAPHY—There are two methods of orthography of the metric units in use. In the original French, the units are spelled me*tre*, li*tre*, gram*me*; in the method proposed by the American Metric Bureau, the units are spelled me*ter*, li*ter*, and gram. For three decades after the original adoption of the metric system, the USP and NF adopted me*ter* and li*ter*, but used the French gram*me*. Now these official compendia use the spelling *gram*.

READING—Some difficulty usually is experienced by those unfamiliar with the metric system in reading the quantities. In the linear measures in pharmacy, centimeters and millimeters are used almost exclusively; thus, 0.05 m would not be read five hundredths of a meter, but rather 5 centimeters (5 cm); if the millimeter column contains a unit, as in 0.055 m, it is read 55 millimeters (55 mm) in preference to fifty-five thousandths of a meter.

Fractions of a millimeter must be read decimally, as 0.0555 m, fifty-five and five-tenths millimeter (55.5 mm). In measures of capacity, cubic centimeters (cc) or milliliters (mL) are used exclusively for quantities of less than a liter. The terms half-liter, quarter-liter, 100 milliliters, and 1 milliliter are denoted by 500 mL, 250 mL, 100 mL, and 1 mL; with water the milliliter is considered equivalent to a gram.

In weight, when the quantity is relatively large and in commercial transactions, the *kilogram* is abbreviated to *kilo*. When less than a *kilogram* and not less than a *gram*, the quantity is read with the gram for the unit. Thus, 2000 g would be read either as 2000 grams or as 2 kilos, and 543 g would be read 543 grams; 2543 g is sometimes read 2 kilos and 543 grams, although 2543 grams usually is preferred.

For quantities below the *gram*, decigram and centigram usually are not used, but rather *milligram* has been regarded as the most convenient unit. With the increase in the use of extremely small doses of very potent drugs and the wide application of more delicate analytical procedures, the term *microgram* (mcg, μg, or γ), for thousandths of a milligram, is used frequently to designate quantities up to 999 μg (less than 1.000 mg).

Both the metric and English systems of weights and measures are in use in the US. Even though the metric system nearly has replaced the English system, the pharmacist must have a practical knowledge of both.

WEIGHTS

The Metric System

The USP of 1890 adopted the metric system of weights and measures to the exclusion of all others except for equivalent dosage statements, and the British Pharmacopoeia of 1914 did likewise. In 1944 the Council on Pharmacy and Chemistry of the American Medical Association adopted the metric system exclusively. The advantages of the metric or decimal system, and its simplicity, brevity, and adaptability to everyday needs are now conceded universally.

FRACTIONAL AND MULTIPLE PREFIXES—In many experimental procedures, including some in the pharmaceutical sciences, very small (and occasionally very large) quantities of weight, length, volume, time, or radioactivity are measured. To avoid the use of numbers with many zeros in such cases, the NIST recognizes prefixes to be used to express fractions or multiples of the International System of Units (SI), which was established in 1960 by the General Conference on Weights and Measures (see the foregoing discussion). The recognized prefixes, which in use are adjoined to an appropriate unit (as, for example, in such quantities as nanogram, picomole, microcurie, microsecond, or megavolt) are defined in Table 11-1.

Table 11-2 lists some metric weights. The prefixes, which indicate multiples, are of Greek derivation: deka, 10; hecto, 100; kilo, 1000. Fractions of the units are expressed by Latin prefixes: deci, 1/10; centi, 1/100; milli, 1/1000.

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Table 11-1. Prefixes for Fractions and Multiples of SI Units

FRACTION	PREFIX	SYMBOL	MULTIPLE	PREFIX	SYMBOL
10^{-1}	deci	d	10	deka	da
10^{-2}	centi	c	10^2	hecto	h
10^{-3}	milli	m	10^3	kilo	k
10^{-6}	micro	μ	10^6	mega	M
10^{-9}	nano	n	10^9	giga	G
10^{-12}	pica	p	10^{12}	tera	T
10^{-15}	femto	f	10^{15}	peta	P
10^{-18}	atto	a	10^{18}	exa	E

Table 11-2. Metric Weight

1 microgram	μg	=	0.000,001	g
1 milligram	mg	=	0.001	g
1 centigram	cg	=	0.01	g
1 decigram	dg	=	0.1	g
1 gram	g	=	1.0	g
1 dekagram	dag	=	10.0	g
1 hectogram	hg	=	100.0	g
1 kilogram	kg	=	1000.0	g

Note: The abbreviation μg or mcg is used for microgram in pharmacy, rather than gamma (γ) as in biology.

Only a few of the most convenient denominations are employed in practical work. Whole numbers from 1 to 1000 usually are expressed in terms of grams, while the kilogram is used as the unit for larger quantities. Quantities between 1 milligram and 1 gram usually are referred to in terms of milligrams; microgram (μg or mcg) is used in quantitative analysis, biological studies, and for minute dosage statements.

The English Systems

In the US, both the avoirdupois and apothecary systems of weight measurement sometimes are used in handling medicines. It must be emphasized *that pharmacists may buy their drugs by avoirdupois weight*. These two systems differ:

> 1 pound avoirdupois = 7000 gr and is abbreviated lb.
> 1 pound apothecary = 5760 gr and is abbreviated ℔.
> 1 ounce avoirdupois = 437.5 gr and is abbreviated oz.
> 1 ounce apothecary = 480 gr and is abbreviated ℥.

The *grain* avoirdupois is exactly the same as the *grain* apothecary. The apothecary pound is therefore 1240 gr *lighter* than the avoirdupois pound, and the apothecary ounce is therefore 42.5 gr *heavier* than the avoirdupois ounce.

The abbreviations of the denominations of apothecary weight are represented by the signs ℥, ounce; ℨ, dram; ℈, scruple; and gr, grain. These long have been in use but possibly may be mistaken for one another in rapid or careless writing. The abbreviations or signs of avoirdupois weight differ from those of apothecary weight, and care should be used not to confound them; they are lb (sometimes written #), pound: oz, ounce: gr, grain. Tables 11-3, 11-4, and 11-5 show three English systems of weight.

Jewelers evaluate precious stones with troy weight, which is very similar to apothecary weight. The apothecary and troy grain, ounce, and pound are identical, but the ounces are subdivided differently. The *carat*, used by jewelers, is equal to 3.168 troy grains or 4 carat grains. When used to express the fineness of gold, 1 carat signifies 1/24 part. A 14-carat ring is 14/24 pure gold.

As indicated in the footnote to Table 11-6, a number of special metric system units are used in various pharmacopeial and nonofficial descriptions, tests, and assays of drugs and other substances to express linear measurements of very small dimension. These units and their symbols or abbreviations are listed in Table 11-7, together with their equivalents in terms of the other metric units and the inch.

Table 11-3. Avoirdupois Weight

POUNDS	OUNCES	GRAINS
1 =	16 =	7000
	1 =	437.5

Note: 2000 lb = 1 ton, and 2240 lb = 1 long ton.

Table 11-4. Apothecary Weight

POUNDS	OUNCES	DRAMS	SCRUPLES	GRAINS
1 =	12 =	96 =	288 =	5760
	1 =	8 =	24 =	480
		1 =	3 =	60
			1 =	20

Table 11-5. Troy Weight

POUNDS	OUNCES	PENNYWEIGHTS	GRAINS
1 =	12 =	240 =	5760
	1 =	20 =	480
		1 =	24

Table 11-6. Metric Linear Measure

1 nanometer	(nm)	= 0.000,000,001 m (0.001 μm: 10^{-9} m: 10 Å)
1 micrometer	(μm)	= 0.000,001 m (0.001 mm: 10^{-6} m: 10,000 Å)
1 millimeter	(mm)	= 0.001 m
1 centimeter	(cm)	= 0.01 m
1 decimeter	(dm)	= 0.1 m
1 meter	(m)	= 1.0 m
1 dekameter	(dam)	= 10.0 m
1 hectometer	(hm)	= 100.0 m
1 kilometer	(km)	= 1000.0 m

Note: Although the meter (m) is observed to be the initial unit, it is seldom necessary to use it in pharmaceutical practice, and the same holds true for a number of the above measures. The micrometer (μm), millimeter (mm), and centimeter (cm) are employed in the description of many official drugs. Measurements pertaining to spectrometric and colorimetric tests and assays of many official drugs are recorded in micrometers (μm) or reciprocal centimeters (cm^{-1}) for infrared and in nanometers (nm) for ultraviolet and visible wavelengths of light, respectively.

Table 11-7. Equivalent Linear Measurements

UNIT	INCHES	mm	μm	nm	Å
1 inch	1	25.4	25,400	2.54×10^7	2.54×10^8
1 mm (millimeter)	0.0394	1	1000	10^6	10^7
1 μm (micrometer)	3.94×10^{-5}	10^{-3}	1	1000	10,000
1 nm (nanometer)	3.94×10^{-8}	10^{-6}	10^{-3}	1	10
1 Å (angstrom unit)	3.94×10^{-9}	0^{-7}	10^{-4}	0.1	1

MEASURES

Systems

Two systems of linear measure are used in the US: English and metric. Two systems of liquid measure are used: apothecary (also called the wine measure or US liquid measure) and metric. The units of the English system of linear measure (inch, foot, yard, mile) are well-known, and needn't be described here. The units of the metric systems of linear and liquid measure, and of the apothecary (wine, US liquid) system of liquid measure, with their respective equivalents, are given in Tables 11-6, 11-8, and 11-9.

Pharmacists who fill Canadian or British prescriptions should also be familiar with the substantially different British imperial liquid measure system; the units, with their equivalents, are given in Table 11-10.

The following facts concerning the US system of liquid measure (see Table 11-9) should be noted:

1. The apothecary fluidounce (f$\mathfrak{z}$) of distilled water weighs 454.6 gr at 25°C (77°F).
2. The apothecary pint contains 16 f$\mathfrak{z}$.
3. The US gallon contains 128 f$\mathfrak{z}$ or 231 inch^3. One gallon of distilled water weighs 8.337 avoir lb at 62°F. The US pint therefore weighs 1.04 avoir lb and the pound of distilled water measures only 0.96 pt. *One pound does not measure 1 pt.*

The following facts concerning the imperial system (see Table 11-10) should be noted:

1. The imperial fluidounce of distilled water weighs 437.5 gr at 15.6°C (60°F). It therefore weighs 1 avoir oz.
2. The imperial pint contains 20 f$\mathfrak{z}$.
3. The imperial gallon contains 160 f$\mathfrak{z}$. One gal of distilled water weighs 10 avoir lb; 16 f$\mathfrak{z}$ in this system therefore weighs 1 avoir lb.

From the above, one can deduce the following:

1. The US fluidounce and minim are larger than the imperial fluidounce and minim (ℳ). One US minim or fluidounce equals 1.04 imperial minims or fluidounces.
2. The imperial pint and gallon are much larger than the US pint and gallon.

It is, therefore, inaccurate to use measuring devices calibrated in the US system in measuring quantities directed in English prescriptions when the imperial measure is intended. Conversely, devices calibrated in the imperial system should not be used to measure quantities directed in US prescriptions when the US measure is intended. For example, Canadian pharmacists using American graduated cylinders should calculate percentage solutions on the basis of 454.6 gr of distilled water to the fluidounce. This is one more argument in favor of adoption internationally by all pharmacists of the metric system of weights and measures.

Table 11-8. Metric Liquid Measure

1 microliter	(μL)	=	0.000001	L
1 milliliter	(mL)	=	0.001	L
1 centiliter	(cL)	=	0.01	L
1 deciliter	(dL)	=	0.1	L
1 liter	(L)	=	1.0	L
1 dekaliter	(daL)	=	10.0	L
1 hectoliter	(hL)	=	100.0	L
1 kiloliter	(kL)	=	1000.0	L

Note: The standard of capacity is the *liter,* which is the volume of 1 kg of distilled water at its maximum density (approx 4°C). Microliters (μL) are used to measure volumes of solutions used in chromatographic procedures for the separation and quantitative determination of some official drugs.

Table 11-9. Apothecary or Wine Measure (US)

GALLON	PINTS	FLUIDOUNCES	FLUIDRAMS	MINIMS
Cong 1 =	8	128	1024	61,440
	1	16	128	7,680
		f$\mathfrak{z}$ 1	8	480
			f$\mathfrak{z}$1	ℳ60

Note: Cong is the abbreviation for the Latin word *congius.*

Table 11-10. Imperial Measure (British)

GALLON	PINTS	FLUIDOUNCES	FLUIDRAMS	MINIMS
Cong 1 =	8	160	1280	76,800
	1	20	160	9,600
		fl$\mathfrak{z}$ 1	8	480
			fl$\mathfrak{z}$ 1	ℳ60

Note: Cong is the abbreviation for the Latin word *congius.* The gill, which is ¼ pint, is obsolete but is found occasionally in old family recipes. Thirty-one US gallons equal 1 barrel.

THE RELATIONSHIPS OF WEIGHTS AND MEASURES

When the systems of weights and measures in use in the US are examined, the lack of close relation between the different units is appreciated at once. Nevertheless, if the following points are used carefully, many pharmaceutical problems will be greatly simplified.

1. Pharmacists may weigh themselves, buy merchandise, sell over the counter, calculate postage, and so on using avoirdupois weight, which contains *437.5 gr in 1 oz.*
2. Pharmacists may compound formulas by apothecary weight, which contains *480 gr in 1 $\mathfrak{z}$.*
3. One apothecary fluidounce of water weighs *455 gr* at 25°C. Since 480 ℳ weigh 455 gr, 1 ℳ weighs 455/480 = 0.95 gr.

    1 ℳ does *not* weigh 1 gr.
    1 f$\mathfrak{z}$ does *not* weigh 1$\mathfrak{z}$

Practical Equivalents

Tables of weights and measures and a table of practical equivalents should be kept in a conspicuous and convenient place in the prescription department, and the following equivalents, which are given with practical accuracy, should be committed to memory. Other equivalents may be calculated from these.

Linear Measure

1 meter	= **39.4 inches**
1 inch	= 2.54 cm = **25.4 mm**
1 micrometer	= **1/1000 mm** = 10^{-6} m = 1/25,400 inch

Liquid Measure

1 milliliter	= **16.2 ℳ**
1 fluidounce	= **29.6 mL**
1 pint	= **473 mL**
1 gallon	= **3790 mL**

Weight

1 kilogram	= **2.20 lb avoir**
1 pound avoir	= **454 g**
1 ounce avoir	= **28.4 g**
1 ounce apothecary	= **31.1 g**
1 pound apothecary	= **373 g**
1 gram	= **15.4 gr**
1 grain	= **64.8 mg**

The USP *Table of Metric Doses with Approximate Apothecary Equivalents* is reproduced in the Appendix, along with information concerning its permissible uses.

Approximate Measures

In apportioning doses for a patient, the practitioner usually is compelled to order the liquid medicine to be administered in certain quantities that have been established by custom, and estimated as:

HOUSEHOLD MEASUREMENT	APOTHECARY NOTATION	METRIC VOLUME
1 tumblerful	f℥viii	240 mL
1 teacupful	f℥iv	120 mL
1 wineglassful	f℥ii	60 mL
2 tablespoonfuls	f℥i	30 mL
1 tablespoonful	f℥iii	15 mL
1 dessertspoonful	f℥ii	8 mL
1 teaspoonful	f℥i	5 mL
½ teaspoonful		2.5 mL

Note: 1 drop is often considered to be 1 minim, but this is incorrect, as drops are variable.

In almost all cases, careful tests have found the modern teacups, tablespoons, dessertspoons, and teaspoons to average 25% greater capacity than the theoretical quantities just given. The physician and the pharmacist therefore should recommend the use of accurately graduated medicine droppers, teaspoons, and calibrated measuring devices, which may be procured at a small cost (Fig 11-1).

Approximate Dose Equivalents

For many years the apothecaries' system of weights and measures was used widely by physicians and pharmacists when considering the doses of medicinal substances, and it was customary to trans-
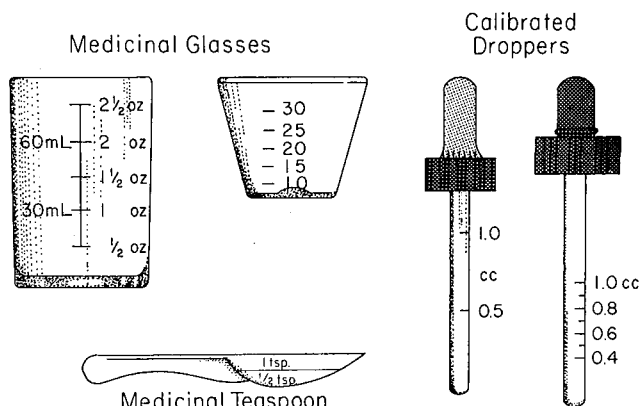
Figure 11-1.

late these apothecary doses into relatively exact amounts when the metric equivalents were mentioned. Today, however, a united effort is being made to establish doses primarily in the metric system and to select for these doses the metric quantities that produce the desired therapeutic effect, without considering the relation of these metric figures to the corresponding quantities in any other system of weights and measures.

It should be emphasized that exact alternative formulas in the avoirdupois system of weights and measures are not obtained by using approximate equivalents but, for the purpose of compounding, should be calculated with the use of practical equivalents.

WEIGHING AND MEASURING

Having studied the several systems of weights and measures, students may now learn to apply their knowledge to the *weighing* and *measuring* of pharmaceuticals. The former process requires the use of the *balance*, or, for manufacturing purposes, *scales*, and the latter process requires the use of the *measure*, the *graduate,* and the *pipet*. The successful performance of many of the operations in pharmacy depends on a thorough knowledge of the principles of the balance and a correct understanding of its care and use; because weighing is nearly always the preliminary step in any compounding, it will be discussed first.

There is a relativity of accuracy in weighing (or measuring) must not be overlooked, as illustrated by the following graded list: coal, salt, sugar, epsom salt, penicillin G, morphine, digoxin, vitamin B_{12}, and radium. One of the most important things for the pharmacist to learn is the degree of tolerance or error permissible in weighing or measuring any particular ingredient. Obviously, the final item on the list, radium, must be measured with much greater precision and accuracy than coal, the first item.

The empiric weighing and measuring methods of the kitchen, embodied in such concepts as a handful, a pinch, or "sweeten to suit your taste," have no place in pharmacy. Accurate work can be accomplished only by means of suitable apparatus.

WEIGHING

In pharmacy, weighing usually refers to ascertaining a definite weight of material to be used in compounding a prescription or manufacturing a dosage form.

The *balance* may be defined as an instrument for determining the relative weights of substances. It should be *selected correctly* for the specific task at hand, *used skillfully*, *protected from damage,* and *checked periodically*, if accurate results are to be obtained. Of even greater importance is its *construction.* Standards for balances are given by the NIST.[1]

Construction of the Balance

For systematic consideration pharmaceutical balances may be classified as follows: single-beam, equal-arm, unequal arm, compound lever, and torsion.

SINGLE-BEAM EQUAL-ARM BALANCES—The principle on which single-beam equal-arm balances (or scales) operate is clearly evident in the construction of the classical two-pan analytical balance. This type has a metallic lever or beam, divided into two equal arms at the center by a knife-edge, on which it is supported. At exactly equal distances from this point of support, and situated in the same plane, are placed the end knife-edges; these suspend the pans, which carry the substances to be weighed. A properly constructed balance of this type should meet the following requirements:

1. *When the beam is in a horizontal position, the center of gravity should be slightly below the point of support, or central knife-edge, and perpendicular to it.*

The relative sensitivity of the balance depends on the fulfillment of this principle, which may be illustrated roughly by forcing a pin through the center of a circular piece of pasteboard. If the edge of the pasteboard is touched slightly, it does not oscillate at all, but rather revolves around the center to a degree corresponding to the impulse given it. In this position it illustrates neutral equilibrium. If the pin is removed and

inserted at a very short distance above the center, and the edge of the pasteboard touched as before, it will oscillate slowly, corresponding to a very sensitive beam, the point of support being slightly above the center of gravity as in the balance. If the pin is removed again and inserted far above the center, and the same impulse imparted to the edge, it will oscillate quickly, illustrating stable equilibrium characteristic of a beam which comes to rest quickly and is not particularly sensitive. Unstable equilibrium may be illustrated by balancing the disc so that the point of support is below the center. The slightest touch then causes it to reverse its position completely and finally come to rest with the center of gravity below the point of support.

2. The end knife-edges must be exactly equal distances from the central knife-edge; they all must be in the same plane, and the edges absolutely parallel to each other.

It is very apparent that the conditions of a good prescription balance cannot be satisfied if there is inequality in the length of the arms of the beam. The distance from the central knife-edge to the one on the left must be exactly the same as the distance from the central knife-edge to the one on the right, otherwise unequal weights would be required to establish equilibrium. If the central knife-edge is placed either above or below a line drawn so that it connects the end knife-edges, the loading of the pans either will cause the beam to cease oscillating or diminish the sensitivity in proportion to the load. If the knife-edges are not parallel, the weight of a body will not be constant upon every part of the pan, but will be greater if placed near the edge on one side, and correspondingly less at a point directly opposite.

3. The beam should be inflexible, but as light in weight as possible, and the knife-edges in fine balances should bear upon agate plates.

The rigidity of the beam is necessary because any serious deflection caused by a loading of the pans would lower the end knife-edges and thus accuracy in weighing would be impossible. The beam should not be heavier than necessary because the sensitiveness of the balance thereby would be lessened; to diminish friction, which constantly increases with the age and use of a balance, the bearings of the knife-edges should be agate plates, which are polished flat pieces of the very hard mineral called agate.

A single-beam equal-arm balance with two rider beams, one graduated to 10 g in increments of 0.1 g, and the other to 200 g in increments of 10 g, is shown in Figure 11-2.

UNEQUAL-ARM BALANCES—The unequal-arm balance is the type is preferred for laboratory work when large amounts are to be weighed (Fig 11-3). The lever principle on which these scales are constructed is based on the law of physics that at equilibrium the force applied at one end of the lever multiplied by the length of the arm (distance from the fulcrum to the point where the force is applied) must be equal to the product of the force acting at the opposite end of the lever and the
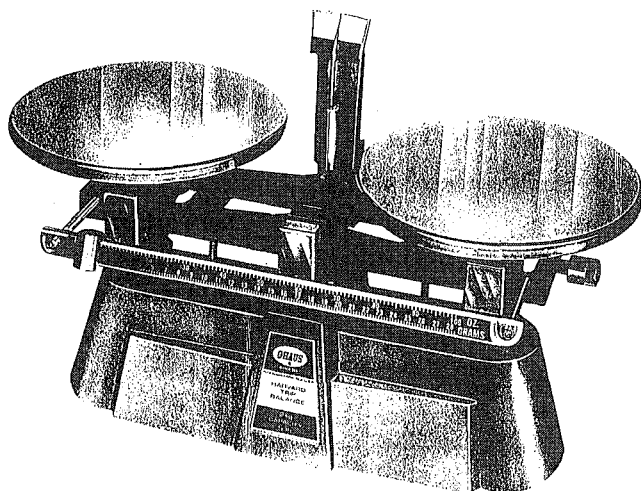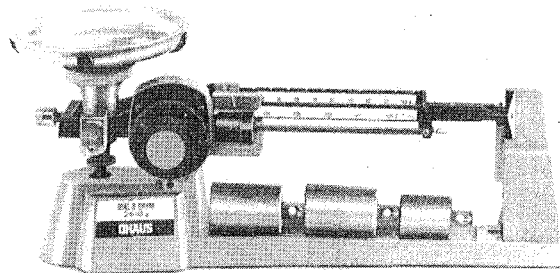
Figure 11-3. Manufacturing laboratory scale and weights (courtesy Ohaus).

length of the other arm. The inequality in the length of the arms of this beam permits the convenient use of movable weights upon the graduated longer arm of the beam, thus dispensing with the use of small weights, which are liable to be lost. This scale is of great advantage in laboratory or manufacturing work because it is particularly adapted for weighing liquids; a sliding tare is set on one beam for the weight of the container, and other sliding weights can be adjusted to the weight of liquid desired. These are available with the beams graduated either in the avoirdupois or metric system.

COMPOUND-LEVER BALANCES—The principle of the compound lever was first applied in the construction of balances by Robervahl of Paris, in about 1660 AD. It was skillfully adapted for both prescription balances and the general counter and platform scales. The principal objection to this type of scale, when compared with single-beam balances, consists in the multiplicity of points of contact and suspension, thus necessarily increasing friction and the liability to disarrangement; however, their general convenience has made them popular.

TORSION BALANCES—A simple illustration of the principle of torsion is afforded by tying a stout piece of cord to a firm support and inserting a lead pencil in the middle of the cord between the strands, at right angles to it. If the free end of the cord is stretched tightly, resistance is offered to any effort to turn the lead pencil over; if the pencil is released, it at once flies back to its original position. *Torsion* is the term applied to this method of twisting. The principle of supporting the beam of a balance on a tightly stretched wire, with the view of doing away with knife-edges and diminishing friction, occupied the attention of inventors for years.

In 1882 Prof Roeder and Dr Springer contrived an ingenious torsion balance that gave promise of valuable results. Two illustrations of this original balance were shown on page 54 of the first edition of *Remington's Practice of Pharmacy* in 1885. Improvements have increased its efficiency greatly. The most important difficulty in applying the principle of torsion resistance was overcome by placing a weight just above the center of gravity. Torsional resistance tends to keep the beam in a horizontal position, while the elevation of a weight above the center of gravity, by its tendency to produce unstable equilibrium, exercises an opposite effect—the beam is inclined to be top heavy and, therefore, to tip on either side. If now the weight is made adjustable by mounting it upon a perpendicular screw so that it can be raised or lowered, it is possible to arrange these opposite forces so that one exactly neutralizes the other. In this manner sensitivity is obtained.

The torsion principle has been applied to prescription balances, as well as analytical balances and scales designed to carry heavier loads. In the torsion prescription balance two beams are used, supported on three frames, each of the latter having a flattened metallic band stretched tightly over its edge.

The torsion balance, which has a rider beam graduated upon the upper edge from ⅛ to 15 gr and on its lower edge from

Figure 11-2. Single-beam equal-arm balance (courtesy Ohaus).

0.01–1.0 g, furnishes a very convenient means of weighing small quantities without having to use small weights. Most modern balances have a direct-reading dial instead of a rider beam, with the metric scale on the upper scale and the apothecary scale on the lower.

The prescription balance may be placed upon a base containing a drawer that can be used for holding weights or powder papers.

Prescription Balances

The modern type of prescription balance uses the taut-wire frame or torsion principle (Fig 11-4). Such balances, manufactured to meet the requirements of the NIST Class III balances, have a maximum maintenance sensitivity of 6 mg with no load and with full load; ie, addition of the 6 mg weight to one pan causes the indicator or the rest point to be shifted not less than one division on the index plate. The Class III balance is used to weigh quantities up to 60 g, depending on the stated capacity and subject to the physical limit of the amount of the material that can be placed on the pan. All prescription departments must have a Class III balance.

REQUIREMENTS—A prescription balance should meet the following general requirements:

1. It should be constructed so as to support its full capacity without developing undue stresses, and should not be thrown out of adjustment by repeated weighings of the capacity load. (The capacity of the balance will be seen on the metal plate attached to it.) If the capacity is not stated, it is assumed to be at least 15 g (½ oz). The Class III balances usually have a capacity of 60 g (2 oz).
2. The removable pans of a prescription balance should be of equal weight. If the pans show any difference in weight, they should be adjusted by leveling the balance or using small pieces of paper. Pans with any appreciable corrosion or wear should be refinished or replaced.
3. A prescription balance should have a leveling device, usually leveling feet or screws, so that the balance can be adjusted to a level position. A balance that does not have these is not entitled to be designated as a prescription balance.
4. The balance that has a rider or graduated dial should have, at the end of the graduation, a stop that halts the rider or dial at the zero reading. The reading edge of the rider should be parallel to the graduations on the beam.
5. The indicator points, when there are two on the balance, should be sharp, and their ends should not be separated by more than 1 mm (0.04 inch) when the scale is in balance. The distance from the face of the index plate to the indicator pointer or pointers should be small (1 mm or less) to protect the operator against making errors resulting from parallax, because it is unlikely that the eye of the

operator will be exactly in line with the indicator and the division on the index plate. The indicating elements as well as the lever system of the balance should be protected against drafts. The balance should have a lid that allows a weighing to be made when the lid is closed.
6. A prescription balance must have a mechanical means for arresting the oscillation of the mechanism.

TESTING—Certain tests may be used to satisfy the user regarding the construction and character of a balance when its origin, history, or condition is in doubt. Additional tests are carried out by the NIST, manufacturers, and local and state testing agencies.

A Class III prescription balance meets the following basic tests. Use a set of *test weights* and keep the rider or graduated dial at zero unless directed to change its position.

1. **Sensitivity Requirement**—Level the balance, determine the rest point, place a 6-mg weight on one of the empty pans, and again determine the rest point. Repeat the operation with a 10-mg weight in the center of each pan. The rest point is shifted not less than one division of the index plate each time the 6-mg weight is added.
2. **Arm Ratio Test**—This test is designed to check the equality of length of both arms of the balance. Determine the rest point of the balance with no weight on the pans. Place 30 g of test weights in the center of each pan and determine the rest point. If the second rest point is not the same as the first, place a 20-mg weight on the lighter side; the rest point should move back to the original place on the index plate scale or farther.
3. **Shift Tests**—These tests are designed to check the arm and lever components of the balance.

a. Determine the rest point of the indicator without any weights on the pans.
b. Place one of the 10-g weights in the center of the left pan, and place the other 10-g weight successively toward the right, left, front, and back side of the right pan, noting the rest point in each case. If in any case the rest point differs from the rest point determined in (a), add the 6-mg weight to the lighter side; this should cause the rest point to shift back to the rest point determined in (a) or farther.
c. Place a 10-g weight in the center of the right pan, and place a 10-g weight successively toward the right, left, front, and back sides of the left pan, noting the rest point in each case. If in any case the rest point is different from that obtained with no weights on the pans, this difference should be overcome by addition of the 6-mg weight to the lighter side.

A balance that does not measure up to these tests *must* be corrected.

4. **Rider- and Graduated-Dial Tests**—Determine the rest point for the balance with no weight on the pans. Now place on the left pan the 500-mg test weight and move the rider to the 500-mg point on the beam. Now determine the rest point. If it is different from the zero rest point, add a 6-mg weight to the lighter side. This should bring the rest point back to its original position or farther. Repeat this test, using the 1-g test weight and moving the rider or graduated dial to the 1-g division. If the rest point is different it should be brought back at least to the zero rest point position by the addition of 6 mg to the lighter pan. If the balance does not meet this test, the graduated beam or the rider must be corrected. For balances equipped with a dial scale, the dial must be corrected.

PROTECTION—The necessity for protecting the delicate mechanism of a balance is overlooked frequently, notwithstanding the possibility of having a precision apparatus irretrievably ruined by lack of care in using or cleaning it or in protecting it while at rest. The position chosen for the balance or scales should be on a level and firm counter, desk, or table, where it will be subjected to little risk of damage from dampness, dust, or corrosive vapors and where the knife-edges will not be liable to become dulled by jarring or other vibrations.

In the analytical class of balances, protection is afforded by enclosing them in glass cases having sash doors in the front, sides, or back. They are protected against damage from vibration by a lever for elevating or locking the beam, so that the knife-edges are not in contact with any surface when not in use. To prevent damage from jarring while the balance is in use, from a weight falling on the pan, or other accident, the finest balances are provided with pan supports, which break the fall
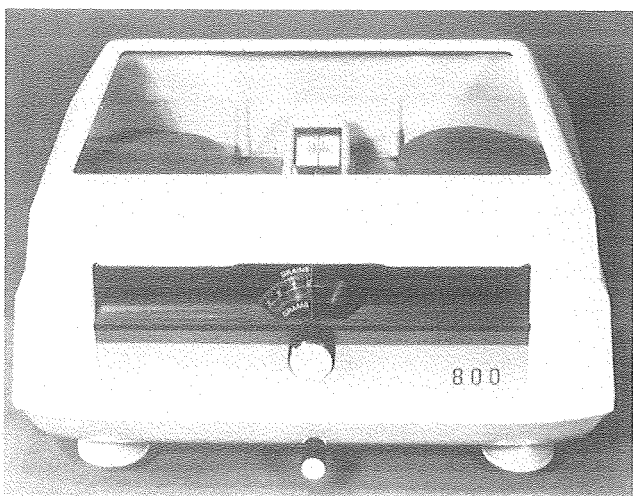

Figure 11-4. Troemner/800 prescription balance (courtesy Troemner).

and serve the additional purpose of quickly arresting the beam, thus saving time while weighing.

In using a prescription balance, neither the weights nor the substance that is to be weighed should be placed on the balance pans while the beam is free to oscillate. The desired weight should be placed upon one pan (usually the one on the right-hand side) and an amount of the substance to be weighed, approximately the desired weight, upon the opposite pan. The beam should be released by means of the lever, and if the substance is in excess, the beam should be locked and a small portion removed and the beam again released and the oscillations observed. This procedure should be repeated until the correct amount is obtained. In case of a deficiency of the substance to be weighed, the reverse procedure is followed until the correct amount is obtained. With practice this can be done very deftly and very quickly and the sensitivity of the balance retained for years.

Substances that react with metals, such as iodine, and those that are adhesive, such as the extracts, should not be weighed directly upon the pans, but rather upon counterpoised watch crystals, or upon glazed paper, care being taken to balance the papers before weighing the substance. In cleaning the balances, great care should be exercised; polishing powders should be used sparingly, as a portion is very apt to find its way into crevices and elude detection until an attempt is made to adjust the balances, when the increased weight of one of the sides of the beam leads to its discovery. Frequent cleaning with soft leather generally is sufficient to keep a balance in good order, but once neglect makes it necessary to use more active measures, some simple polishing powder for the metal work, soapsuds for the nickel plate, and simple brushing for the lacquered brass are all that is necessary.

As the pans are subjected to more wear and tear than any other part of the balance, it is economical to use *solid* rather than *plated* pans because constant friction wears off the plating and the additional cost for replating soon absorbs the difference in price. Equipped in this way, and with agate bearings, a prescription balance is durable and really inexpensive because it will remain fully equal to the most exacting demands for a long time.

Weights Used in Pharmacy

The weights used by the pharmacist are very important, and care in their selection and examination is necessary. False economy must be avoided, as the use of cheap, inaccurate weights ultimately leads to serious consequences. Official inspectors have found pharmacies using prescription weights that were so worn that the characters on their faces had disappeared; also, weights have been found with bits of hardened extract and dirt almost entirely obscuring their characters. An unused set of standard weights should be kept on hand so that at least once a year the weights in daily use can be tested and adjusted or rejected if necessary. The standard weights should be used also when the balance is tested. The set should contain the following weights in a well-fitted box with forceps: two 20-g or two 30-g, two 10-g, one 5-g, two 2-g, one 1-g, one 500-mg, one 20-mg, and one 10-mg, all adjusted to NIST tolerances for analytical or Class P weights.

METRIC WEIGHTS—For weighing larger quantities, japanned iron metric weights are available. They are preferably hexagonal, to distinguish them from the round avoirdupois weights. Sets of brass weights, usually in the range of 10 g to 1000 g, fitted into holes of appropriate size in a block of plastic (*block weights*), are especially convenient for many weighing operations. For prescription compounding, accurate sets of weights ranging from 10 mg to 50 g are available. A set containing both metric and apothecary weights is shown in Figure 11-5.

For analytical purposes, metric weights are used exclusively; usually, the highest weight is 100 g, the lowest 1 mg.
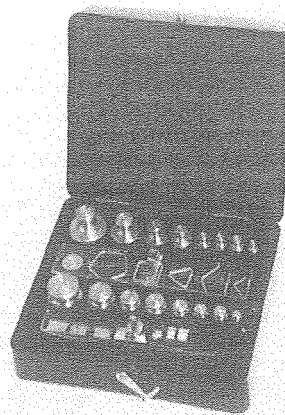
Figure 11-5. Metric and apothecary weight set (courtesy Troemner).

The weights from 1 g upward are of finely lacquered brass or of nonmagnetic stainless steel or rhodium-plated bronze. The smaller weights are made of squares of platinum or aluminum foil, with one edge turned up to permit them to be handled easily with the forceps. Fractions of a milligram are weighed by means of the rider on the graduated beam of the balance.

In analytical work and in using the Class III balance in prescription work, the weights should never be handled with the fingers but always with the forceps, which accompany an accurate set of weights. In the more expensive sets of weights the forceps are tipped with bone, ivory, or plastic to prevent the wearing away of the weights during handling. With proper care the accuracy of a fine set of weights may be maintained for years.

COMMON AVOIRDUPOIS WEIGHTS—Avoirdupois weights usually are made of iron, and they are flat and circular and japanned to prevent rusting. These weights form a pyramidal pile, and range from ½ oz to 4 lb; if found to be incorrect, they may be adjusted by adding to or diminishing the amount of lead that is hammered into a depression in the base of each weight. They sometimes are made of brass in this form, and sometimes of zinc (the latter, however, are brittle and unserviceable). For general use in the pharmacy, the cylindrical weights, known technically as block weights, are preferable. The advantages of block weights are that the gaps left by missing weights are readily noticeable, and the greater part of the surface of the weight is protected from the action of corrosive vapors when the weights are not in use.

APOTHECARY WEIGHTS—Apothecary weights may be obtained either as *block weights* or in the less-desirable *flat* forms. The round, flat, brass *dram* weights, which have the denomination stamped on their faces in raised characters, still are used but should be replaced. With flat weights, the denomination is often only faintly stamped on the face and thus is liable to be obliterated by constant use or by corrosive contact.

Undoubtedly, the best grain weights are the aluminum wire weights. The wire weights are less susceptible to corrosive action than are the brass weights. Also, the wire weights are more easily and quickly distinguished from one another than are other weight forms, so there is less likelihood of dangerous mistakes: the number of sides in the wire weights at once gives the denomination (Fig 11-6).
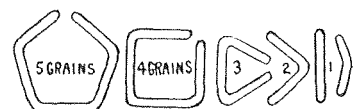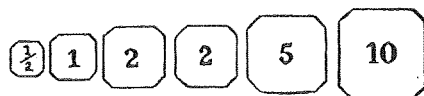

Figure 11-6. Aluminum wire weights.

Figure 11-7. Aluminum grain weights.

Aluminum grain weights, which are cut out of aluminum plates, are also less liable to be corroded. They usually can be more accurately adjusted than brass weights. The corners of the aluminum weights are clipped, and each weight usually is pressed into a curved form so that it may be picked up easily (Fig 11-7).

MEASURING

In pharmacy, *measuring* usually refers to the exact determination of a definite volume of liquid. Many types of apparatus are used in this operation, depending on the kind and quantity of liquid to be measured and the degree of accuracy required. (The NIST has requirements for graduates.[2])

Large Quantities

Glass measures are preferred for measuring liquids. Although glass measures are subject to breakage, they can indicate volume more accurately because of the transparency of glass.

THE MENISCUS—When an aqueous or alcoholic liquid is poured into a graduate, surface forces cause its surface to become concave—the portion in contact with the vessel is drawn upward. This phenomenon is known as the formation of a *meniscus* (Fig 11-8), and in determining the volume of a liquid *the reading must be made at the bottom of this meniscus*. This regulation has been established by the NIST, and all glass measuring vessels are graduated on this basis. Liquids with large contact angles, such as mercury, form an *inverted meniscus,* and the reading then is made at the top of the curved surface.

PROCEDURE—Pharmaceutical manufacturers package liquid preparations in glass or plastic containers equipped with a plastic screwcap. These containers serve as a stock bottle from which liquids may be poured directly into a graduate. The procedure for pouring liquid from screwcapped containers is as follows:

1. Remove the cap and place it on the counter while the transfer of liquid is made.
2. While holding the graduate in the left hand, grasp the original container with the label in such a position that any excess of liquid will not soil the label if it should run down the side of the bottle.
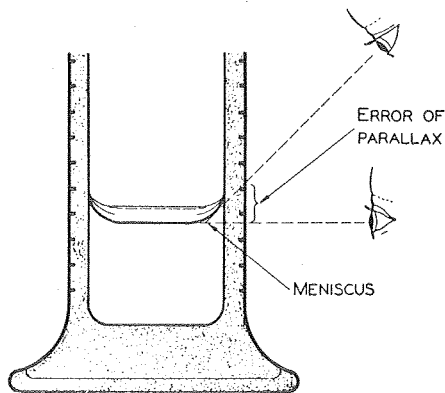
3. Raise the graduate and hold it so that the graduation point to be read is on a level with the eye, and measure the liquid. (The extension of the graduating mark into a circle that passes entirely around the graduate is an improvement that obviates the necessity of placing the graduate upon a level place, as the corresponding mark upon the opposite side may be seen through the glass and the graduate easily leveled even when held in the hand.)
4. Replace the cap, and return the bottle to the counter or shelf.
5. Pour the liquid into the bottle or mortar for dispensing or compounding.

METALLIC MEASURES—Metallic measures are nearly cylindrical in shape, but are slightly wider at the bottom. These are generally used for measuring liquids when the quantity is over a pint. A set usually consists of five (gallon, half-gallon, quart, pint, and half-pint) of these measures. Measures made of tinned iron, or of the enameled sheet iron called agateware, are greatly inferior to those made of *tinned copper* or *stainless steel;* tinned-iron measures soon become rusty, and particles of enameling can chip off, leaving the exposed iron to contaminate the measured liquids.

The initial cost of copper or stainless-steel measures is greater than tinned iron, but they are far more durable. Care must be taken to protect them from blows that will cause dents as these may be serious enough to detract from their accuracy. Cylindrical metric measures, usually made of monel metal or stainless steel and having a diameter just half their height, are available in various sizes. Such containers are relatively expensive, but their resistance to corrosion and wear is a tremendous advantage. Copper, of course, should not be used where it is likely to catalyze oxidation.

GRADUATED GLASS MEASURES—Graduated glass measures nearly always are used for quantities of 500 mL or 1 pt or less. There are of two forms, *conical* and *cylindrical* (Fig 11-9, 11-10). The conical graduate is suitable for some measurements because of the greater ease with which it can be handled, but cylindrical measures are more accurate because of their uniform and smaller average diameter. In a graduated cylinder, the error in volume caused by a deviation of ±1 mm in reading the meniscus remains constant along the height of the

Figure 11-9. Glass conical graduate (courtesy Kimble Glass).

Figure 11-8. Error of measurement due to parallax.

Figure 11-10. Glass cylindrical graduate (courtesy Kimble Glass).

uniform column; the same deviation causes a progressively larger error in a conical graduate because the diameter, and thus the volume of the 1-mm column, increases along its vertical axis. It is safe to assume that practically all good-grade modern graduates comply with the NIST requirements for internal diameters at stated volumes.

A study has indicated that, to improve accuracy, the lower portions of graduates should not be used, and therefore should not be marked.[2] A composite tabulation (Table 11-11) shows the calculated and the assigned blank portions of graduates. The elimination of the lower markings on graduates was suggested, and in 1955 the NIST specifications for graduates used this principle.[1] The NIST Handbook states, "A graduate shall have an initial interval that is not subdivided, equal to not less than one-fifth and not more than one-fourth of the capacity of the graduate." For accurate measurement of volumes less than 1.5 mL, a graduated pipet or a graduated dropper could be used.

EFFECT OF LIQUID AND CONTAINER—It is difficult to measure accurately when pouring from a completely filled bottle because of the uneven flow of the liquid. After the first portion of the liquid is removed, the shape of the bottle does not influence the ease of pouring to any appreciable extent unless the neck is extremely narrow.

Viscous liquids pour slowly, but their accurate measurement is not difficult. Experiments showed that when glycerin is poured into a graduate without letting the liquid run down the inside surface, the precision of measurement can be very high. Naturally, the chance of hitting the inner surface is greater with smaller than with larger graduates. The increase in possible deviation then is caused by the slow movement of the viscous liquid to the desired mark.

Viscous liquids introduce another factor: drainage time. Graduates are calibrated to contain or deliver indicated volumes within specified limits. Aqueous, alcoholic, and hydroalcoholic liquids can be drained from a graduate in 0.5 min so completely that the delivered and contained volumes are fairly close. When 25 mL of glycerin was measured in the same cleaned and dried cylinders, the received volume measured 23.7 mL after the same time period. Silicone-treated glassware, which now is used frequently, drains completely in a few seconds.

The viscosity factor might be altered when another liquid is to be mixed with the glycerin by measuring and mixing both liquids in a suitable graduate.

Small Quantities

For measuring smaller quantities of liquids, graduated glass tubes of small diameter should be used. The narrower bore permits greater distances between the graduations on the apparatus, thus allowing greater accuracy in making the reading. For example, with a buret the pharmaceutical chemist can estimate volumes to the nearest 1/100 mL.

Pipets and similar apparatus are more accurate and convenient than very small graduates. The graduations on very small graduates are necessarily in the very small, lowest portion of a comparatively tall measure. To measure 1 mL or 10 ℞ of a volatile oil in a graduate, the surface that the oil must traverse when this measure is inverted is so great that probably 20% of the oil will be left adhering to the measure. In liquid preparations in which the smaller liquid is miscible with the larger quantity of diluting liquid, the graduate may be rinsed and this loss recovered, but inconveniences are largely overcome and greater accuracy secured by using a pipet.

In administering small quantities of liquids, the very convenient *drop* is almost always used. It should be emphasized that *1 drop is not equivalent to 1* ℞ and that *60 drops are not equivalent to 1 f℥*. This impression doubtlessly arose because 60 ordinary drops of *water* are about equal to 1 f℥, but the volume of a drop of fluid depends on many factors, including density, temperature, viscosity, surface tension, and the size and nature of the orifice from which it is dropped. Thick, viscous liquids, such as the mucilages and the syrups, necessarily produce large drops because the drop adheres to the surface of the glass as long as its weight does not overcome its power of adhesion, whereas chloroform, a mobile liquid that has very little adhesion to the dropping surface, produces very small drops. The greater the surface tension, the larger the drop, and the greater the extent of surface to which the drop adheres, the larger, proportionally, the drop.

A *normal or standard drop measure* was recommended by the Brussels Conference of 1902 for international adoption. This dropper is recognized in the USP.

MEDICINE DROPPER[3]

The Pharmacopeial medicine dropper consists of a tube made of glass or other suitable transparent material that generally is fitted with a collapsible bulb and, while varying in capacity, is constricted at the delivery end to a round opening having an external diameter of about 3 mm. The dropper, when held vertically, delivers water in drops each of which weighs between 45 mg and 55 mg.

When drops are specified on a prescription, the usual custom has been to employ an *eyedropper,* but now the standard dropper should be supplied. When accuracy is required, it is particularly important to use the standard or a specially calibrated dropper for administering potent medicines. The volume error incurred in measuring any liquid by means of a calibrated dropper should not exceed 15%, under normal conditions.[3]

A *standard teaspoon* has not yet received universal acceptance; however, it is generally accepted to be equivalent to 5 mL.

TEASPOON

Agreement has not been reached on a standard official teaspoon, in spite of the need for such a standard measure in connection with compounding and labeling liquid medicines. For household purposes, an American Standard Teaspoon has been established by the American National Standards Institute (1430 Broadway, New York, NY 10018) as containing 4.93 ± 0.24 mL. In view of the almost universal practice of employing teaspoons ordinarily available in the household for the administration of medicine, the teaspoon may be regarded as representing 5 mL.

It must be kept in mind that the actual volume delivered by a teaspoon of any given liquid is related to the latter's viscosity and surface tension, among other influencing factors.

THE HUMAN FACTOR—The *human factor of carefulness* is of paramount importance in every pharmaceutical operation in which accuracy is essential. Accurate measurement of liquids requires accurate equipment, careful manipulation, good vision, and a steady hand.

Table 11-11. Unmarked (Unreliable) Portions of Graduates

| CAPACITY OF GRADUATE (mL) | CALCULATED BLANKS (1951) | | NBS BLANKS (1965) (mL) |
	2.5%[a] ALLOWED (mL)	5%[a] ALLOWED (mL)	
5	3.0	1.5	1
10	4.4	2.2	2
25	11.8	5.9	5
50	15.8	7.9	10
100	20.9	10.5	20
250	36.3	18.2	50
500	66.5	33.2	100
1000	—	—	200

[a] Calculations by Goldstein and Mattocks[2] based on deviation of ±1 mm from graduation mark and allowable errors of 2.5 and 5%.

DENSITY AND SPECIFIC GRAVITY

Several terms are used to express the mass (weight) of equal volumes of different substances.

Absolute density is the ratio of the mass of an object, determined in or referred to a vacuum, at a specified temperature, to the volume of the object at the same temperature. This relationship is expressed mathematically as:

$$\frac{\text{Mass in grams (in a vacuum)}}{\text{Volume in millimeters}} = \text{Absolute density}$$

Apparent density differs from absolute density only in that the mass of the object is determined in air; the mass is influenced by the difference in the buoyant effect of air on the object being weighed, and on the standard masses (weights) used for comparison. If the object and masses are made of the same material, or have the same density, there will be no difference in the buoyant effect, and the apparent density will be identical with the absolute density.

Relative density is an expression sometimes employed to indicate the mass of 1 mL (not cc, which is very slightly different) of a standard substance, such as water, at a specified temperature, relative to water at 4°C taken as unity. Thus, at 4°C the relative density of water is 1.0000, whereas its absolute density at the same temperature is 0.999973. Water attains its maximum absolute density of 0.999973 at 3.98°C. To convert a relative density of water to absolute density, the former should be multiplied by 0.999973.

Specific gravity may be defined as the ratio of the mass of a substance to the mass of an equal volume of another substance taken as the standard. For gases, the standard may be hydrogen or air; for liquids and solids, it is water.

From what has been stated, it is obvious that in a determination of specific gravity there will be, in general, a difference in the result if the masses (weights) are determined in air or in vacuum. If the masses are determined in, or referred to, a vacuum, the result is a *true specific gravity* (sometimes called *absolute specific gravity*); if the masses are determined in air, the calculated result is an *apparent specific gravity*. The difference between these specific gravities is, as a rule, very small.

A very important variable in specific gravity determinations is temperature, and this is doubly important because both the temperature of the substance under examination and the temperature of the standard may be different. The temperatures are commonly shown as a ratio, with the temperature of the water always being indicated in the denominator. The common practice with regard to the determination of specific gravity is that defined by the USP: "Unless otherwise stated, the specific gravity basis is 25°/25°, ie, the ratio of the weight of a substance in air at 25° to that of an equal volume of water at the same temperature."

But it is not always convenient, or desirable, to determine the weight of both the substance and the water at 25°, or even to determine the weight of the substance at the same temperature as that at which the water is weighed. Thus, the substance may be weighed at 25°, and compared with the weight of an equal volume of water at 4°, in which case the specific gravity is reported as being on a 25°/4° basis. In the case of theobroma oil, which is solid at 25°, the specific gravity is determined on a 100°/25° basis; for alcohol, it is determined on a 15.56°/15.56° basis because many years ago the US government adopted 60°F (15.56°C) as the temperature at which alcoholometric measurements are to be made for government control of alcoholic liquids.

It is apparent that a completely informative statement of specific gravity must indicate the temperature of the substance under examination, as well as that of the equal volume of water. Furthermore, it should be stated whether the determinations of mass (weight) were made on an *in-vacuum* or *in-air* basis; the latter case, the material of construction of the weights also should be indicated (as the buoyant effect of air on weights depends on their volume).

Calculations

The principle underlying the determination of the specific gravity of either a liquid or a solid is the same: to find the ratio of the mass (weight) of the substance to that of an equal volume of water. This may be expressed by a simple relationship:

$$\text{Specific gravity} = \frac{W_s}{W_w}$$

where W_s is the weight of the substance, and W_w the weight of an equal volume of water.

DENSITY

Density is defined as the mass of a substance per unit volume. It has the units of mass over volume. *Specific gravity* is the ratio of the weight of a substance in air to that of an equal volume of water. In the metric system both density and specific gravity may be numerically equal, although the density figure has units. In the English system, density and specific gravity are not numerically equal; for example, the density of water is 62.4 lb/ft^3 and the specific gravity is 1. This shows the convenience of the metric system. The equations for calculating density, weight, and volume are

$$\text{Density} = \frac{\text{Weight}}{\text{Volume}}$$

$$\text{Weight} = \text{Density} \times \text{Volume}$$

$$\text{Volume} = \frac{\text{Weight}}{\text{Density}}$$

Given any two variables, the third one can be calculated.

Examples

1. A pharmacist weighs out 2 kg of glycerin (density, 1.25 g/mL). What is the volume of the glycerin?

$$\text{Volume} = \frac{2000 \text{ g}}{1.25 \text{ g/mL}} = 1600 \text{ mL}$$

2. What is the weight of 60 mL of an oil whose density is 0.9624 g/mL?

$$\text{Weight} = 60 \text{ mL} \times 0.9624 \text{ g/mL}$$
$$= 57.7 \text{ g}$$

3. Calculate the weight of 30 mL of sulfuric acid (density, 1.8 g/mL).

$$\text{Weight} = 1.8 \text{ g/mL} \times 30 \text{ mL} = 54 \text{ g}$$

4. If a prescription order requires 25 g of concentrated hydrochloric acid (density, 1.18 g/mL), what volume should the pharmacist measure?

$$\text{Volume} = \frac{25 \text{ g}}{1.18 \text{ g/mL}} = 21.2 \text{ mL}$$

Problems (Answers on page 122)

1. What is the weight in grams of 1 L of alcohol (density, 0.816 g/mL)?

2. What is the volume (mL) of 1 lb (avoir) of glycerin (density, 1.25 g/mL)?

3. What is the volume (mL) of 65 g of an acid whose density is 1.2 g/mL?

Pharmaceutical dispensing and compounding calculations use simple arithmetic. The errors that may arise often are due to carelessness, as in improper placing of decimal points, incorrect conversion from one system of measurement to another, or uncertainty over the system of measurement to be used. Before proceeding with any calculation it is imperative that the problem presented (in a prescription, chart order, formula, etc) be read carefully, that the information given and required be identified, and that the procedure to be used in the calculation be selected.

Before students read this part of the chapter and attempt to solve the problems, the information in the preceding part of this chapter must be understood thoroughly. Often, several steps are necessary to solve problems. Shortcuts should not be taken unless one is certain they are proper. Many problems can be solved by more than one procedure, such as by ratio and proportion or by dimensional analysis. If students find a procedure that is more logical to them and gives the correct answer, it should be used. Thus, the solutions to sample problems used here generally should be considered suggestions, rather than the only way to solve a given type of problem.

A few mathematical principles (eg, common decimal fractions, exponents, powers and roots, significant figures, and logarithms) will be reviewed, as these are areas where students often become careless or have forgotten skills. Following this, various types of practical pharmaceutical problems that the pharmacist may be required to solve are discussed and solutions are given. Where practical, rules for solving these problems are given. No attempt is made to elaborate on any mathematical theory.

The problems generally consist of determining the quantity or quantities of material(s) required to compound prescriptions properly and make products used to aid the compounding of prescriptions. The materials used to compound prescription orders may be pure or mixtures of substances in varying strengths. The strengths of mixtures may be denoted in different ways: Conversions may be necessary between systems of varying strengths or between different measuring systems. At the end of each section, sample problems are given for the student to solve, the answers to which appear on page 119.

Because of the decreasing importance of the apothecary system, the metric system is emphasized here. Chemicals and preparations most likely will be purchased using the avoirdupois or metric systems. Prescription orders are filled in the system indicated on the order, usually the apothecary or metric systems.

The student should become familiar with the terminology used in writing prescription orders, such as Latin words and abbreviations used in giving directions to the pharmacist and patient (see Chapter 97). The prescriber occasionally may use Roman numerals instead of Arabic numerals, so students must be familiar with these (even if the practice is declining).

SIGNIFICANT FIGURES

Weighing and measuring can be carried out with only a certain maximum degree of accuracy; the result always is approximate due to the many sources of error such as temperature, limitations of the instruments employed, personal factors, and so on.

Pharmacists must achieve the greatest accuracy possible with their equipment, but it would be erroneous to claim that they have weighed 1 mg of a solid on a Class III prescription balance, which has a sensibility reciprocal of 10 mg, or that they have measured 76.32 mL of a liquid in a 100-mL graduate, which can be read only to 1 mL. When quantities are written, the numbers should contain only those digits that are *significant* within the precision of the instrument.

Significant figures are digits that have practical meaning. In some instances zeros are significant; in other instances they merely indicate the order of magnitude of the other digits by locating the decimal point. For example, in the measurement 473 mL all the digits are significant, but in the measurement 4730 mL the zero may or may not be significant. In the weight 0.0316 g the zeros are not significant but only locate the decimal point. In any result the last significant figure is only approximate, but all preceding figures are accurate. When 473 mL is recorded, it is understood that the measurement had been made within ± 0.5 mL or somewhere between 472.5 and 473.5 mL. The student should stop to consider the full implications of this, specifically that the measurement is subject to a maximum error of:

$$\frac{0.5}{473} \times 100 = \text{(approx) } 0.1\% \text{ or } 1 \text{ part in } 1000$$

A zero in a quantity such as 473.0 mL is a significant figure and implies that the measurement has been made within the limits 472.95 mL and 473.05 mL or with a possible error of:

$$\frac{0.05}{473} \times 100 = 0.01\% \text{ or } 1 \text{ part in } 10,000$$

Thus, 473 is correct to the nearest mL, and 473.0 is correct to the nearest 0.1 mL.

Rules

1. *When adding or subtracting, retain in the sum or remainder no more decimal places than the least number entering into the calculations.* For example,

	11.5 g		11.50 g
	2.65 g		2.65 g
	3.49 g		3.49 g
	17.64 g		17.64 g
Answer:	17.6 g	*Answer:*	17.64 g

In the first column 11.5 g was weighed to 0.1 g or with an accuracy of ±0.05 g. Although the other two weighings were made with an accuracy of ±0.005 g, the sum can be expressed properly only to one decimal place.

In the second column 11.50 g was weighed to the nearest 0.10 g or with an accuracy of ±0.005 g. Since all weighings were made with this degree of accuracy, the sum may be stated as in the example, 17.64 g.

Retain all figures possible until all the calculations are completed and then retain only the significant figures for the answer. Additions or subtractions involving both large and small quantities, each expressed with maximum significance, are often useless. For example, if one were to add 1.2 and 0.041 g, the physical sum would be 1.2 g, regardless of the fact that the two numbers add numerically to 1.241. To express the physical sum as 1.241 g would convey an erroneous degree of accuracy with which the quantity was known.

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Table 11-12.

WEIGHT		EQUIVALENT WEIGHT (gr/g)		EQUIVALENT WEIGHT (gr)	SIGNIFICANT FIGURES
4.522	×	15.432	=	69.78	4
4.522	×	15.43	=	69.77	4
4.522	×	15.4	=	69.6	3
4.522	×	15	=	68	2

2. When multiplying or dividing, retain in the answer no more significant figures than the least number entering into the calculation.

The meaning of this rule may be illustrated by the use of equivalents during conversions from one measuring system to another. Table 11-12 gives different equivalent values and the number of significant figures to which the answer is correct. Always use an equivalent which will give the desired degree of accuracy. Repeated multiplication of an approximation increases the error progressively; therefore, retain all figures during calculations and drop insignificant figures as the final step.

FRACTIONS

Common Fractions

An example of a common fraction is 3/8. It is read as "three-eighths" and indicates three parts divided by eight parts of the same thing. The units with both numbers must be the same. Pharmacists measure 3/8 of a fluidounce into a graduate, they measure 3 fluidrams, out of 8 fluidrams (a fluidounce contains 8 fluidrams).

The following principles should be applied when using common fractions:

1. The value of a fraction is not altered by multiplying or dividing both numerator and denominator by the same number.
2. Multiplying the numerator or dividing the denominator by a number, multiplies the fraction by that number.
3. Dividing the numerator or multiplying the denominator by a number divides the fraction by that number.
4. To add or subtract fractions, form fractions with the *lowest common denominator*, perform the arithmetical operation, and reduce to the lowest common denominator.
5. To multiply fractions, multiply all numbers above the line to form the new numerator and multiply all numbers below the line to form the new denominator. Cancel if possible to simplify and reduce to the lowest common denominator.
6. To divide by a fraction, multiply by the reciprocal of the fraction.

Decimal Fractions

Fractions with the power of 10 as the denominator are known as *decimal fractions* and are written by omitting the denominator and inserting a decimal point in the numerator as many places from the last number on the right as there are ciphers of 10 in the denominator.

The following principles should be applied when using decimal fractions:

1. When adding or subtracting decimals, align the decimal points under each other.
2. When multiplying decimals, proceed as with whole numbers, then place the decimal point in the product as many places from the first number on the right as the sum of the decimal places in the multiplier and the multiplicand.
3. When dividing by a decimal fraction, move the decimal point to the right, in both divisor and dividend, as many places as it is to the left in the divisor to form a whole number in the divisor; proceed as with whole numbers. The decimal point in the quotient should be placed immediately above the decimal point in the dividend.

4. When converting a common fraction into a decimal fraction, divide the numerator by the denominator and place the decimal point in the correct place.
5. When converting a decimal fraction into a common fraction, place the entire number, as the numerator, over the power of 10 containing the same number of ciphers of 10 as there are decimal places. Cancel, if possible, to simplify.

EXPONENTS, POWERS, AND ROOTS

In the expression $2^4 = 16$, the following names are given to the terms: 16 is called the *power* of the *base 2*, and 4 is the *exponent* of the power. If the exponent is 1, it usually is omitted. The following laws should be recalled:

1. The product of two or more powers of the same base is equal to that base with an exponent equal to the sum of the exponents of the powers; eg, $2^5 \times 2^3 = 2^8$.
2. The quotient of two powers of the same base is equal to that base with an exponent equal to the exponent of the dividend minus the exponent of the divisor; eg, $2^8 \div 2^3 = 2^5$.
3. The power of a power is found by multiplying the exponents; eg, $(2^8)^3 = 2^{24}$.
4. The power of a product equals the product of the powers of the factors; eg, $(2 \times 3 \times 4)^2 = 2^2 \times 3^2 \times 4^2$.
5. The power of a fraction equals the power of the numerator divided by the power of the denominator; eg,

$$\left(\frac{2}{3}\right)^2 = \frac{2^2}{3^2}$$

The root of a power is found by dividing the exponent of the power by the index of the root; eg,

$$\sqrt[3]{3^6} = 3^{6/3} = 3^2$$

Any number other than 0 with an exponent 0 equals 1; eg, $2^0 = 1$. A number with a negative exponent equals 1 divided by the number with a positive exponent equal in numerical value to the negative exponent; for example,

$$2^{-4} = \frac{1}{2^4}$$

To facilitate the solution of involved and lengthy problems, *logarithms (logs)* were invented. Many calculations that are difficult by ordinary arithmetical processes are performed rapidly and easily with the aid of logs. The log of a number is the exponent of the power to which a given base must be raised in order to equal that number.

$$Y = a^x$$

$$\log_a Y = x$$

John Napier, of Scotland, who discovered logs over three centuries ago, used the Natural Log Number, 2.71828+, as the base. Henry Briggs, using Napier's discovery a few years later, introduced 10 as the base, which is the most convenient for practical purposes. Napier's system is called natural logs and Briggs' system is called common logs. In this latter system the natural numbers are regarded as powers of the base 10 and the corresponding exponents are the logs; eg,

$$100 = 10^2$$

$$\log_{10} 100 = 2$$

$$2 = 10^{0.3010} \text{ or } \log_{10} 2 = 0.3010$$

For natural logs,

$$6 = e^{1.792}$$

$$\ln_e 6 = 1.792$$

LAWS AND RULES

The following laws, governing the use of logs, are based on the laws of exponents, and hence hold for any log system.

1. The log of a product equals the *sum* of the log of the component numbers; for example, for 25×2:

$$\log (25 \times 2) = \log 25 + \log 2$$

$$= \log 10^{1.3979} + \log 10^{0.3010}$$

$$= 1.3979 + 0.3010 = 1.6989$$

2. The log of a quotient equals the log of the numerator minus the log of the denominator; for example, for $25 \div 2$:

$$\log (25 \div 2) = \log 25 - \log 2 = \log 10^{1.3979} - \log 10^{0.3010}$$

$$= 1.3979 - 0.3010 = 1.0969$$

3. The log of a power of a number equals the log of the number multiplied by the exponent of the power; for example, for $(25)^{12}$:

$$\log (25)^{12} = 12 \log 25 = 12 \times 1.3979 = 16.7748$$

4. The log of a root of a number equals the log of the number divided by the index of the root; for example, for $\sqrt{25}$

$$\log \sqrt{25} = \log 25^{1/2} = \frac{\log 25}{2} = \frac{1.3979}{2} = 0.6990$$

5. The log of a negative power of a number equals the reciprocal of the number multiplied by the exponent of the power; for example, $(5)^{-2}$:

$$\log (5)^{-2} = -2 \log 5 = -2 \times 0.6990 = -1.398$$

The logs of 1, 10, 0.01, and so on are integers, but for numbers between these the logs will consist of two parts: an integral part called the *characteristic* and a fractional part called the *mantissa*. Thus,

$10^2 = 100$	$\log 100 = 2$
$10^1 = 10$	$\log 10 = 1$
$10^0 = 1$	$\log 1 = 0$
$10^{-1} = 0.1$	$\log 0.1 = -1$
$10^{-2} = 0.01$	$\log 0.01 = -2$

The log of a number between 100 and 1000 has 2 for a characteristic plus a fraction, the log of a number between 0.1 and 0.01 has −2 for a characteristic plus a decimal, and so on. The mantissa of a log always must be positive, whereas the characteristic may be either positive or negative.

Every number may be regarded as the product of two numbers, one being 10 with a positive or negative exponent and the other being some number between 1 and 10; eg,

$$760 = 10^2 \times 7.6 = 10^2 \times 10^{0.8808}$$

$$\therefore \log 760 = \log 10^2 + \log 10^{0.8808} = 2.8808$$

$$0.076 = 10^{-2} \times 7.6 = 10^{-2} \times 10^{0.8808}$$

$$\therefore \log 0.076 = \log 10^{-2} + \log 7.6 = -2 + 0.8808$$

This is written $\bar{2}.8808$ (or $8.8808 - 10$).

The characteristic is made a positive number by subtracting the −2 from 10 to give a characteristic of 8 . . . −10. The −10 is put after the mantissa. From the above explanation the following rules are derived:

1. The characteristic of a number greater than 1 is one unit less than the number of figures to the left of the decimal point; eg, for 1000 the characteristic is 3.
2. The characteristic of a number less than 1 is one unit more than the number of ciphers between the decimal point and the first significant figure; eg, for 0.001 the characteristic is −3.
3. If the characteristic of a log is positive, the integral part of the corresponding number contains one more figure than the number of units in the characteristic; eg, if the characteristic equals 2, the corresponding number lies between 100 and 1000.
4. If the characteristic of a log is negative, the number of zeros between the decimal point and the first significant figure is one less than the number of units in the characteristic; eg, if the characteristic is −2, the corresponding number lies between 0.01 and 0.001.
5. Numbers that are related to each other by some power of 10 possess logs with the same mantissa; eg, log 760 = 2.8808 and log 76 = 1.8808.

The Log of a Number

The characteristic of a log is determined readily by inspection of the natural number, but to obtain the mantissa a table of logs must be used. These tables vary in accuracy according to the number of decimal places to which the mantissa is expanded. For most calculations four places are satisfactory.

Under the heading *Natural Numbers* (N) in the *Table of Logarithms* (see Appendix), the first two figures of the number are given down the column on the left, while the third figure (from 0 to 9) is given across the top. The mantissa for large numbers or numbers falling between three-place ones may be found by the process of interpolation; eg,

1. Find the log of 273.

Under N find 27 and along the top line find the third number, 3. Across from 27 and under 3 the mantissa for 273 (4362) is found. No interpolation is necessary. By inspection (see rule 1) the characteristic is 2. Then log 273 = 2 + 0.4362 = 2.4362.

2. Find the log of 0.08206.

Under N find 82 and along the top find the next number, 0. Now 8206 falls between 820 and 821 (6/10 of the difference). The mantissa for 820 is 9138 and the mantissa for 821 is 9143. The difference between these two mantissas is 5, and 6/10 of 5 is 3. The mantissa for 8206 is therefore 9138 + 3 = 9141. By inspection (see rule 2) the characteristic is −2. Then log 0.08206 = −2 + 0.9141 = 8.9141 − 10 or $\bar{2}.9141$.

The process of finding a number between two other numbers is known as *interpolation*. It is based on the assumption that the mantissa varies directly with the number, but this is not quite true. Many log tables supply the proportionate parts to facilitate interpolation.

The Antilog of a Number

To find the number corresponding to a given log, the reverse procedure of that discussed above is employed. The first step is to find figures corresponding to the mantissa (interpolation may be necessary). The last step is to place the decimal point in the correct position, following rules 3 and 4; eg,

1. Find the number corresponding to the log 3.8357.

In the log table, 8357 is found across from 68 and under 5. The figures required are therefore 685. Since the characteristic is 3 (rule 3), the log 3.8357 is the number 6850.

2. Find the number corresponding to the log 0.4351.

In the log table, 4351 is found to fall between 4346 and 4362, the difference being 16.4351 is 5 units more than 4346, or 5/16 of the difference between the two mantissas. The log table gives 272 as the antilog of 4346, to which 5/16 or 0.31 must be added. Adding on the 0.3 to the fourth place, the required figures are 2723. Since the characteristic is zero, the required number is 2.723.

The Antilog of a Negative Number

Finding the antilog of a negative number is easy when you remember that the mantissa is always *positive*. Thus, the first step is to convert the negative mantissa to a positive one; eg, $\log X = -3.523$.

1. Add -1 to the characteristic so that it becomes -4.
2. Add $+1$ to the mantissa so that it becomes 0.477 ($+1.0000 - 0.523 = +0.477$).
1. The result is

$$\log X = \bar{4}.477$$

From the log table the antilog of 0.477 is 3.0, so that the antilog of $\bar{4}.477$ is 3.0×10^{-4}. Hence, if $\log X = -3.523$, $X = 3.0 \times 10^{-4}$; eg,

1. Using the Henderson-Hasselbalch equation for an acidic substance, find the ratio of ionized to un-ionized drug at a pH of 3.0. The pK_a of the drug is 7.4.

$$pH = pK_a + \log \frac{[Salt]}{[Acid]}$$

$$pH - pK_a = \log \frac{[Salt]}{[Acid]}$$

$$3.0 - 7.4 = -4.4 = \bar{5}.6 = \log \frac{[Salt]}{[Acid]}$$

$$3.98 \times 10^{-5} = \frac{[Salt]}{[Acid]}$$

LOGARITHMIC CALCULATIONS

Representative problems illustrated below show the rapidity and simplicity of calculations with logs.

1. Find the value of $8.52 \times 36.4 \times 0.0056$.

To multiply, add logs of the numbers.

$$\begin{aligned}
\log 8.52 &= 0.9304 \\
\log 36.4 &= 1.5611 \\
\underline{\log 0.0056} &= \bar{3}.7482 \\
\log number &= 0.2397
\end{aligned}$$

To find the natural number corresponding to log number 0.2397, take the antilog.
    Answer: antilog 0.2397 = 1.737.

2. Find the fifth root of 0.00475.

To find the nth root of a number, divide the log of the number by the index of the root.

$$\log (\sqrt[5]{0.00475}) = \tfrac{1}{5} \log 0.00475 = \tfrac{1}{5} (\bar{3}.6767)$$

$$= \tfrac{1}{5} (7.6767 - 10) = 1.5353 - 2 \text{ or } \bar{1}.5353$$

To find the natural number corresponding to the log number 1.5353, take the antilog.
    Answer: antilog $\bar{1}.5353$ = 0.343.
3. Find the value of

$$\frac{6.062 \times 10^{23}}{0.08206 \times 293.1 \times 760,000}$$

Remember: To multiply, add the logs of the numbers; to divide, subtract the logs of the numbers:

$\log 6.062$	$= 0.7826$	$\log 0.08206$	$= \bar{2}.9141$
$\log 10^{23}$	$= 23.$	$\log 293.1$	$= 2.4670$
$\log$ numerator	$= 23.7826$	$\log 760.000$	$= 5.8808$
		$\log$ denominator	$= 7.2619$

Log value: $23.7826 - 7.2619 = 16.5207$.
    Answer: antilog $16.5207 = 3.32 \times 10^{16}$.

4. The pH of a solution is the log of the reciprocal of the hydrogen-ion concentration. If the concentration of H^+ ions in a solution is 2.57×10^{-4} g-ion/L, what is the pH?

$$pH = \log \frac{1}{[H^+]} = \log \frac{1}{2.57 \times 10^{-4}} = \log \frac{10^4}{2.57}$$

Taking logs

$$pH = \log 10^4 - \log 2.57 = 4 - 0.4099 = 3.59$$

Problems

1. The rate of creaming of an emulsion may be calculated by Stokes' law:

$$-V = \frac{2gr^2(d_2 - d_1)}{9\eta}$$

If $d_1 = 0.88$ g/mL, $d_2 = 1.32$ g/mL, $g = 980.6$ cm/sec^2, $r = 10^{-3}$ cm, and $\eta = 1.14$ poise, find the rate, V.

2. The surface tension (S) of a liquid may be found by the Capillary Rise Method using the formula

$$S = \tfrac{1}{2} hdgr$$

where h is the height of the liquid in the capillary, d is the density of the liquid, g is the acceleration of gravity, and r is the radius of the capillary. Find S when $h = 2.62$ cm, $d = 2.43$ g/mL, $g = 980.6$ cm/sec^2, and $r = 0.021$ cm.

3. Using the Henderson-Hasselbalch equation, find the ratio of un-ionized to ionized drug at a pH of 1.5. The pKa of the basic drug is 9.6.

The student who knows algebra, has studied the previous sections of this chapter, and recognizes the Roman numerals and Latin abbreviations used on prescription orders (for directions to the pharmacist and patient by the prescriber) should have sufficient knowledge to solve the routine problems encountered in a pharmacy. The various symbols and abbreviations and their meanings must be well understood. Explanation of practical problems, representative of those faced in practice, are presented below. Practice problems follow each section and the answers to these problems are found at the end of this chapter (page 122).

To solve each problem properly, the following procedure is suggested:

1. Analyze the problem carefully so that all data are clearly fixed in the mind; determine what is given and what is asked for.
2. Select the most direct method of solving the problem. Not all problems can be solved properly in one step. Look up doses, equivalents, and abbreviations when you are not sure.
3. Prove or check the result.

ADDITION

Review weighing and measuring systems discussed earlier in this chapter. The expression "weighable or measurable quantities" means pounds, ounces, drams, quarts, pints, fluidounces, and so on. For example, it is not practical to weigh 300 gr or measure 50 fl oz, because neither a 300-gr weight nor a 50-fl oz graduate is commonly available. These are converted to

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5 drams, and 1 qt, 1 pt, 2 fl oz, respectively, which are weighable and measurable quantities.

Rules

1. Add like quantities. Using the metric system, if the quantities are not alike, change them to a common unit. Using the apothecary or avoirdupois systems, create columns of like quantities arranged in descending order of magnitude toward the right.

2. In the apothecary or avoirdupois systems, add together the smaller quantities first, then advance to the next higher units.

3. Always extract the next higher unit, wherever possible, to simplify the answer, which should be stated in weighable or measurable quantities.

4. When adding decimals, keep the decimal points directly under each other.

5. When adding fractions, reduce to the lowest common denominator (LCD), add the resulting numerators, and reduce the fraction, if possible, by canceling.

Examples

1. Add 3 kg, 33 g, and 433 mg.

Convert to a common unit. The gram is convenient because it is the unit of weight.

$$3 \text{ kg} = 3 \times 1000 \text{ g} = 3000 \quad \text{g}$$
$$33 \text{ g} = 33 \quad \text{g}$$
$$433 \text{ mg} = 433 \text{ mg} \div 1000 = 0.433 \text{ g}$$
$$\overline{\hspace{2cm} 3033.433 \text{ g}}$$

2. Add 4 pounds, 3 ounces, 1 dram, 59 grains and 5 pounds, 10 ounces, 7 drams, 2 grains (apoth).

lb	℥	ʒ	gr
4	3	1	59
5	10	7	2
9	13	8	61

Explanation:

61 grains = 1 dram + 1 grain (60 grains = 1 ʒ)

Add 1 dram to the next column:

8 + 1 = 9 drams = 1 ounce + 1 dram (8 ʒ = 1 ℥)

Add 1 ounce to the next column:

13 + 1 = 14 ounces = 1 pound + 2 ounces (12 ℥ = 1 ℔)

Add 1 pound to the next column:

9 + 1 = 10 pounds.

Answer: 10 ℔, 2 ℥, 1 ʒ, 1 gr.

3. Add the following volumes: 5 gal, 3 pt, 2 fl oz; and 2 pt, 3 fl oz, 4 fl dr.

Write out in proper sequence of the units in the measuring system and arrange the numbers given in the problem under each other. Thus,

gal	pt	fl oz	fl dr
5	3	2	
	2	3	4
5	5	5	4

Note: 5 pt = 2 qt + 1 pt (2 pt = 1 qt).
Answer: 5 gal, 2 qt, 1 pt, 5 fl oz, 4 fl dr.

Problems

1. Add 25 mg, 25 g, 210 mg, 2 kg, 1.75 g, 215 mg, 454 g, and 30 mg.

2. The following quantities of a drug were removed from a container: 31 g, 225 g, 855.6 g, and 45.4 g. What is the total weight removed from the container?

3. What is the weight of powder formed by mixing together 1 ℥, 175 gr of Drug A, 87.5 gr of Drug B, and 6 ℥, 55 gr of Drug C? Give the answer in weighable quantities.

4. Add ℥xi, ʒvi, Ͽii, gr xiv and ℥vii, ʒv, Ͽii, gr x. Give the answer in weighable quantities.

5. Each unit of a mixture contains the following drugs: 1/5 gr of Drug M, 1/90 gr of Drug N, 1/6 gr of Drug P, and 2½ gr of Drug Q. What is the total weight of each unit?

6. The inventory card shows the following amounts of a syrup: 3 gal, 2½ qt, 6 pt, 8 fl oz, 19 fl oz. What is the total volume in stock (in measurable quantities)?

SUBTRACTION

Rules

1. Subtract only like quantities. If the quantities are not alike, change to a common unit (metric system) or place in columns of like quantities or units arranged in descending order of magnitude toward the right (avoirdupois and apothecary systems).

2. In the apothecary and avoirdupois systems, begin with the smallest quantities and advance to the largest.

3. When necessary, reduce larger quantities to smaller ones and place in the proper column.

4. Treat common and decimal fractions as indicated in the section on addition.

Examples

1. Subtract 1 pt, 4 fl oz, and 6 fl dr from 2 gal.

The problem may be solved as follows: divide 1 gal into 4 qt, leaving 1 gal in its column; divide 1 of the 4 qt into 2 pt, leaving 3 qt; divide 1 pt into 16 fl oz, leaving 1 pt; divide 1 fl oz into 8 fl dr, leaving 15 fl oz.

gal	qt	pt	fl oz	fl dr
1	3	1	15	8
		1	4	6
1	3	0	11	2

Answer: 1 gal, 3 qt, 0 pt, 11 fl oz, 2 fl dr.

2. Subtract 285 mL from 1 L. Convert to a common unit.

$$\begin{array}{r} 1000 \text{ mL} \\ -285 \text{ mL} \\ \hline 715 \text{ mL} \end{array}$$

Answer: 715 mL.

Problems

1. How much is left in a 5-L container after the removal of 895 mL?

2. A pharmacist buys 1 oz of Drug C. At intervals she uses the following quantities to compound prescription orders: ʒii, ʒss, Ͽii, 56 gr, and 48 gr. How much of Drug C remains?

3. A bottle contains 1 pt of a liquid; 8 fl oz and 6 fl dr were removed. How much of the liquid remains?

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4. A pharmacist buys 5 g of a potent drug and at different times dispenses 0.2 g, 0.85 g, 90 mg, and 150 mg on prescription orders. How much of the drug remains?

MULTIPLICATION

Rules

1. The product has the same denomination as the multiplicand.
2. If the multiplicand is composed of different denominations in the metric system, form a common unit before multiplying and reduce the product to measurable units. In the apothecary or avoirdupois systems, arrange the quantities in descending order of magnitude toward the right, and multiply. Extract the next higher units, beginning with the smallest unit, and place in the proper columns, proceeding to the left.
3. Multiply fractions and decimals as in any arithmetic problem, and reduce fractional quantities to measurable or weighable units.

Examples

1. Multiply 4 pt, 7 fl oz and 3 fl dr by 4.
Begin with the smallest unit, working from right to left. When it becomes necessary, change the product to the next higher unit, writing only the remainder, if there is any, under the unit multiplied as

pt	fl oz	fl dr
4	7	3
		×4
16	28	12

12 fl dr = 1 fl oz + 4 fl dr remainder

28 fl oz + 1 fl oz = 29 fl oz = 1 pt + 13 fl oz remainder

16 pt + 1 pt = 17 pt = 2 gal + 1 pt remainder

Answer: 2 gal, 1 pt, 4 fl dr.

2. What will be the total weight of the ingredients in a prescription order for 25 units, each unit containing 0.4 g of Solid F, 0.01 g of Solid G, and 5 mg of Solid H? First, convert to a common unit such as grams.

0.4 g + 0.01 g + 0.005 g = 0.415 g total weight of 1 unit

0.415 g/unit × 25 units = 10.375 g total weight of all units

3. Multiply 22.4 mL by 2.65.

$$\begin{array}{r} 22.4 \text{ mL} \\ \times\ 2.65 \\ \hline 59.36 \text{ mL} \end{array}$$

Problems

1. Multiply 48.5 mL by 3.24.
2. A certain preparation is to contain 0.0325 g of a chemical in each mL of solution. How much must be weighed out to make 5 L of the solution?
3. How much cod liver oil is necessary to make 2500 capsules, each containing 0.33 mL?
4. A formula calls for 1 pt, 3 fl oz, 4 fl dr of an oil. How much is required to make 15 times the formula quantity? Give amounts in measurable quantities.
5. How many mg are used to make 1500 units, each of which contains 250 μg of a drug?

DIVISION

Rules

1. The quotient always has the same denomination as the dividend.
2. If the dividend is composed of different denominations, form a common unit in the metric system before dividing and reduce the quotient to weighable or measurable quantities. In the apothecary or avoirdupois systems, arrange as explained in the multiplication section; begin division with the largest quantity at the left, convert the remainder, if any, into the next lower units, and add to the next column before proceeding with the division.
3. Treat fractions, and decimals as explained in the multiplication section.

Examples

1. Divide 3 L by 25.

$$3 \text{ L} = 3000 \text{ mL}$$

$$\frac{3000 \text{ mL}}{25} = 120 \quad \text{mL}$$

2. Divide 10 gal, 3 pt, 8 fl oz by 8.

	gal	pt	fl oz
8)	10	3	8

$$\frac{10 \text{ gal}}{8} = 1 \text{ gal} + 2 \text{ gal remainder}$$

$$2 \text{ gal} = 16 \text{ pt}$$

Place 16 pt in the next column.

$$16 \text{ pt} + 3 \text{ pt} = 19 \text{ pt}$$

$$\frac{19 \text{ pt}}{8} = 2 \text{ pt} + 3 \text{ pt remainder}$$

$$3 \text{ pt} = 48 \text{ fl oz}$$

Place 48 fl oz in the next column.

$$48 \text{ fl oz} + 8 \text{ fl oz} = 56 \text{ fl oz}$$

$$\frac{56 \text{ fl oz}}{8} = 7 \text{ fl oz}$$

Answer: 1 gal, 2 pt, 7 fl oz or 1 gal, 1 qt, 7 fl oz.

The alternative method is to reduce all quantities to a small unit such as fl oz, then divide and convert to measurable quantities.

(10 gal × 128 fl oz/gal) + (3 pt × 16 fl oz/pt) + 8 fl oz = 1336 fl oz

$$\frac{1334 \text{ fl oz}}{8} = 167 \text{ fl oz}$$

Extract the largest units possible (convert to measurable quantities).

167 fl oz	39 fl oz
−128 fl oz = 1 gal	−32 fl oz = 2 pt = 1 qt
39 fl oz remainder	7 fl oz remainder

Answer: 1 gal, 1 qt, 7 fl oz.

3. A pharmacist buys an 8-oz container of a drug. How many 5-gr capsules can be made from the contents?

a. The pharmacist usually purchases by the avoirdupois system. The first step is to convert ounces to grains.

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$$437.5 \text{ gr/oz} \times 8 \text{ oz} = 3500 \text{ gr}$$

b. Since 3500 gr are available and each capsule contains 5 gr, divide the total amount by 5 gr.

$$\frac{3500 \text{ gr}}{5 \text{ gr}} = 700$$

Therefore, 700 5-gr capsules can be made.

Problems

1. How many 65-mg capsules can be made from 50 g of a drug?

2. How many 15-minim capsules can be filled from 5 fl oz of an oil?

3. The dose of a drug is 0.1 mg. How many doses are contained in 15 mg of the drug?

4. The dose of a drug is 1/150 gr. How many doses are obtainable from 1 gr of the drug?

5. How many 325-mg capsules of a drug can be filled from a 454-g amount?

CONVERSION

As long as the student knows the interrelationships of the various units within the different weighing and measuring systems (eg, 20 gr = 1 Э, 3 Э = 1 Ʒ; 1000 mg = 1 g), there are only three conversions necessary to memorize in order to convert between the apoth, avoir, and metric systems. These are

$$1 \text{ gr (avoir)} = 1 \text{ gr (apoth)}$$

$$15.4 \text{ gr} = 1 \text{ g}$$

$$16.2 \text{ ℳ} = 1 \text{ mL}$$

Learn them!

With these three conversions the student is able to derive all other necessary conversions.

Apothecary Conversions

Various equalities within the apothecary system may be calculated. The number of grains in a dram, grains in a pound, and so on may be calculated using the following steps.

1.

$$20 \text{ gr/Э} \times 3\text{Э/Ʒ} = 60 \text{ gr/Ʒ}$$

$$60 \text{ gr/Ʒ} \times 8\text{Ʒ/Ʒ} \times 12\text{Ʒ/lb} = 5760 \text{ gr/lb}$$

Cancel the units. If they do not cancel properly, something has been omitted.

2.

$$1 \text{ gr (apoth)} = 1 \text{ gr (avoir)}$$

Since 1 gr (apoth) = 1 gr (avoir), the number of grains in one system equals the number of grains in the other system; eg, 480 gr (apoth) = 480 gr (avoir).

Convert 1 Ʒ (apoth) to weighable quantities in the avoir system

$$20 \text{ gr/Э} \times 3\text{Э/Ʒ} \times 8\text{Ʒ/Ʒ} = 480 \text{ gr/Ʒ} \text{ (apoth)}$$

$$480 \text{ gr (apoth)} = 480 \text{ gr (avoir)}$$

$$437.5 \text{ gr} = 1 \text{ oz avoir}$$

$$\begin{array}{r} 480.0 \text{ gr} \\ -437.5 \text{ gr} \\ \hline 42.5 \text{ gr} \end{array}$$

Answer: 1 Ʒ (apoth) = 1 oz, 42.5 gr (avoir).

3. Conversions in the metric system are made in the same manner.
Convert 1 g to mg.

$$1 \text{ g} \times \frac{1000 \text{ mg}}{\text{g}} = 1000 \text{ mg.}$$

Convert 1 g to kg.

$$1 \text{ g} \times \frac{1 \text{ kg}}{1000 \text{ g}} = 0.001 \text{ kg}$$

The same procedure is valid for volume measurements in the metric system.

4. Conversions between the apothecary and metric weight systems are based on the fact that 15.4 gr = 1 g, which may be restated as 15.4 gr/g or 1 g/15.4 gr.

a. How many mg equal 1 gr?

$$\frac{1.000 \text{ g}}{15.4 \text{ g}} = 0.0648 \text{ g/gr} = 64.8 \text{ mg/gr or } 64.8 \text{ mg} = 1 \text{ gr}$$

Cancel units.

b. How many grams are in 1Ʒ?

$$\frac{1.000 \text{ g}}{15.4 \text{ gr}} \times 480 \text{ gr/Ʒ} = 31.1 \text{ g/Ʒ}$$

c. How many grams are in 1 oz (avoir)? *Remember:* 1 gr (apoth) = 1 gr (avoir).

$$\frac{1.000 \text{ g}}{15.4 \text{ gr}} \times 437.5 \text{ gr/oz} = 28.4 \text{ g/oz}$$

d. Other weight conversions are then found in a similar manner.

5. Conversions between the apothecary and metric measuring systems are based on the fact that 16.2ℳ = 1 mL, which may be restated as 16.2 ℳ/mL or 1 mL/16.2 ℳ.

a. How many mL are in 1 fl oz?

$$60 \text{ ℳ/fl dr} \times 8 \text{ fl dr/fl oz} = 480 \text{ ℳ/fl oz}$$

$$480 \text{ ℳ/fl oz} \times \frac{1 \text{ mL}}{16.2 \text{ ℳ}} = 29.6 \text{ mL/fl oz}$$

$$or\ 29.6 \text{ mL} = 1 \text{ fl oz}$$

Rules

1. The USP states that for prescription compounding one uses exact equivalents rounded to three (3) significant figures.

2. To calculate quantities required in pharmaceutical formulas, the USP directs the use of exact equivalents.

3. In converting doses the USP uses approximate equivalents. Use USP tables wherever possible.

Examples

1. Convert 1 pt, 4 fƷ into mL.

First, convert into fƷ.

$$16 \text{ fƷ/pt} + 4 \text{ fƷ} = 20 \text{ fƷ}$$

Second, convert fƷ to mL.

$$1 \text{ fƷ} = 29.6 \text{ mL (as calculated above)}$$

$$20 \text{ fƷ} \times 29.6 \text{ mL/fƷ} = 592 \text{ mL}$$

Answer: 1 pt, 4 fl oz = 592 mL.

2. What is the weight of 1200 g in the apothecary system?

$$1 \text{ g} = 15.4 \text{ gr}$$

$$1200 \text{ g} \times 15.4 \text{ gr/g} = 18,516 \text{ gr}$$

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Convert to weighable quantities.

$$480 \text{ gr} = 1 \text{ʒ}$$

$$480 \text{ gr/ʒ} \times 38 \text{ʒ} = 18{,}240 \text{ gr}$$

$$\begin{array}{l} 18{,}516 \text{ gr} \\ -18{,}240 \text{ gr} \ (38 \text{ʒ}), \ 38 \text{ʒ} = 3 \text{℔}, \ 2 \text{ʒ} \ (12 \text{ʒ} = 1 \text{℔}) \\ \hline 276 \text{ gr} \\ -240 \text{ gr} \ (60 \text{ gr} = 1 \text{ʒ}), \ (4 \text{ʒ}) \\ \hline 36 \text{ gr} \end{array}$$

Answer: 3 ℔, 2 ʒ, 4 ʒ, 36 gr (apoth).

3. Convert 1 pound (apoth) into grams.

$$\frac{1 \text{ g}}{15.4 \text{ gr}} \times 480 \text{ gr/ʒ} = 31.1 \text{ g/ʒ}$$

$$1 \text{ lb} = 12 \text{ʒ}$$

$$12 \text{ʒ} \times 31.1 \text{ g/ʒ} = 373.3 \text{ g/lb}$$

4. Convert 25 gr to grams.

$$25 \text{ gr} \times \frac{1 \text{ g}}{15.4 \text{ gr}} = 1.62 \text{ g}$$

5. Convert 50 grams to grains.

$$50 \text{ g} \times 15.4 \text{ gr/g} = 770 \text{ gr}$$

Problems

1. Convert:

a. 6.50 grains into milligrams.
b. 3/10 grain into milligrams.
c. 3½ apoth ounces into grams.
d. 2 ʒ into mg.
e. 3½ avoir ounces into grams.
f. 1 lb avoir into grams.

2. Convert:

a. 550 g into weighable quantities in the avoir system.
b. 450 mg into grains.
c. 550 g into weighable quantities in the apoth system.
d. 100 μg into grains.
e. 1 kg into lb (avoir).

3. Convert the following doses into metric weights:

a. 1/100 gr.
b. 1/320 gr.
c. 1/6 gr.
d. 5 gr.
e. 20 gr.

4. Convert:

a. 200 ℳ into mL.
b. 3 fl dr into mL.
c. 8 fl oz into mL.
d. 1 pt into mL.
e. 5 ℳ into mL.
f. 0.1 mg into gr.
g. 5 mg into gr.

5.

a. How many gr are in 1 ʒ?
b. How many drams are in 1 ʒ?
c. How many grains are in 1 oz (avoir)?
d. How many gr are in ½ lb (apoth)?
e. Convert 250 gr to weighable quantities in the apothecary system.

HOUSEHOLD EQUIVALENTS

Common household equivalents are found on page 96. These are used to interpret the prescriber's instructions to the patient. The teaspoonful usually is indicated by the symbol fʒ or 5 mL, although 1 fʒ does not equal 5 mL. The problem of "the teaspoonful" has been discussed by Morrell and Ordway.[4] For practical purposes, a teaspoonful is equal to 5 mL, and 1 fʒ in the directions to the patient on the prescription means 1 teaspoonful; therefore there are 6 teaspoonful quantities in 1 fluidounce (5 mL × 6 = 30 mL).

For purposes of solving most compounding and dispensing problems, the exact equivalents rounded to three significant places should be used.

DOSAGE CALCULATIONS

Over the past years various rules for calculating infants' and children's dosages have been devised. All of them give only approximate dosages because they erroneously assume that the child is a small adult; some of them are still used because as yet no absolute method of calculating an infant's or child's dose has been found. Children are sometimes more susceptible than adults to certain drugs. Doses for infants and children, where they are known, may be found in the USP, and APhA booklet entitled *Pediatric Dosage Handbook* and textbooks on pediatrics.[5-7] Doses should not be calculated when it is possible to obtain the actual infant's or child's dose.

Rules for Approximate Doses for Infants and Children

1. *Young's Rule* (for children 2 years old and older).

$$\frac{\text{Age (years)}}{\text{Age (years)} + 12} \times \text{Adult dose} = \text{Child's dose (approx)}$$

2. *Clark's Rule*.

$$\frac{\text{Weight (lb)}}{150} \times \text{Adult dose} = \text{Child's dose (approx)}$$

3. *Fried's Rule* (for infants up to 2 years old).

$$\frac{\text{Age (months)}}{150} \times \text{Adult dose} = \text{Infant's dose (approx)}$$

4. *The Square Meter Surface Area Method* relates the surface area of individuals to dose. It is thought that this is a more realistic way of relating dosages (see Crawford et al,[8] Talbot et al,[9] and Butler and Richie[10]).

$$\frac{\text{Body surface area of child}}{\text{Body surface area of adult}} \times \text{Adult dose}$$
$$= \text{Child's dose (approx)}$$

The average body surface area for an adult has been given as 1.73 square meters (m²); hence,

$$\frac{\text{Body surface area of child (m}^2\text{)}}{1.73} \times \text{Adult dose}$$
$$= \text{Child's dose (approx)}$$

Calculating Doses for Individuals

The body surface area for individuals may be found in various reference sources such as the previously mentioned APhA

booklet, and in drug dosage data by Shirkey.[11] Talbot et al[9] include a chart that relates weight to body surface area. Wagner[12] presents a discussion on dosage of drugs.

Many drugs have doses stated as the amount of *drug/m²* *body surface area* and may be calculated as follows:

Amount of drugs/m² × Body surface area in m² = Individual's dose

Many physiological functions are proportional to body surface area, such as metabolic rate and kidney function.

Drug doses are often stated in *mg/kg body weight* and may be calculated as follows:

$$mg/kg \times \text{Body weight in kg} = \text{Individual's dose}$$

This is the most common way of determining children's doses.

Drug doses also may be stated in *units,* as with vitamins A and D, penicillin, and hormones. This means that a certain quantity of biological activity of that drug is called 1 unit. When the term unit is used in connection with a drug, the calculations involved are the same as those for more familiar weight or volume notations. The USP often standardizes the unit for such drugs, so the expression "USP Units" is used. This means the units are calculated based on a USP assay procedure and reference standard.

Examples

1. The adult dose of a drug is 5 gr. What is the dose for a 3-year-old child?

Use Young's Rule:

$$\text{Child's dose (approx)} = \frac{3}{3 + 12} \times 5 \text{ gr} = 1 \text{ gr}$$

2. What is the dose for a 40-lb child if the average adult dose of the medicament is 10 mg?

Use Clark's Rule:

$$\text{Child's dose (approx)} = \frac{40}{150} \times 10 \text{ mg} = 2.67 \text{ mg}$$

3. What is the dose for an 8-month-old infant if the average adult dose of a drug is 250 mg?

Use Fried's Rule:

$$\text{Infant's dose (approx)} = \frac{8}{150} \times 250 \text{ mg} = 13.3 \text{ mg}$$

4. If the average adult dose of a drug is 50 mg, what is the dose for a child who has a body surface area equal to 0.57 m²?

$$\text{Child's dose (approx)} = \frac{0.57}{1.73} \times 50 \text{ mg} = 16.5 \text{ mg}$$

Problems

1. What is the dose of a drug for a 9-month-old infant if the average adult dose is 25 mg?
2. What is the dose of a drug for a 6-year-old child if the average adult dose is 1½ gr?
3. What is the dose of a drug for a child who weighs 28 lb if the average adult dose is 100 mg?
4. What is the dose of a drug for an individual who has a 1.21 m² body surface area? The average adult dose is 400,000 units.
5. What is the dose of a medicament for a child that weighs 66 lb if the dose is stated as 2.5 mg/kg body weight?
6. What is the dose of a drug for an average adult patient if the dose of the drug is 45 mg/m²?

PROBLEM-SOLVING METHODOLOGY

Two problem-solving methods will be illustrated in solving pharmaceutical problems: ratio and proportion, and dimensional analysis. *Ratio and proportion* forms the basis for nearly all calculations and is a concept that seems to be generally understood and used. However, many students and pharmacy practitioners have difficulty in problem interpretation and identifying a place to start. *Dimensional analysis* is based on ratio and proportion and offers an alternate way to solve problems and overcome some problem-solving difficulties.

RATIO AND PROPORTION

A *ratio* states the relation of one quantity to another and may be written as a common fraction (implying division) or with a colon between the two numbers. For example, three parts compared with four parts is written 3/4, 3:4, or three is to four. Any units may be substituted for "parts" but the value of the ratio does not change. The units must be the same.

Two equal ratios that are set equal to each other result in an equation a *proportion.* For example, 3/4 = 15/20, 3:4::15:20, or "three is to four as fifteen is to twenty" are ways of writing and stating that 3 and 4 form the same ratio or fraction as 15 and 20.

The first and last terms of a proportion are called the *extremes,* and the second and third terms are called the *means.*
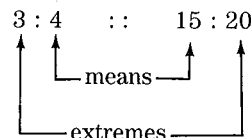
Rules

The following statements are true for any proportion:

1. The product of the means equals the product of the extremes.
2. The product of the means divided by one extreme gives the other extreme.
3. The product of the extremes divided by one mean gives the other mean. Therefore, if any three terms of a proportion are known, the fourth can be found by simple calculation.

In solving problems involving proportions, the following procedure may be used:

1. Let the unknown quantity be represented by X, and let it be the fourth term.
2. Let the third term be that number in the question which expresses the same kind of value (unit) as is expected in the answer.
3. Arrange the remaining two quantities in the same ratio as the third term and X. Thus, the first and second terms will express the same kind of values (units) and the third and fourth terms will express the same kind of values. If the answer sought (X) is to be greater than the third term, the second term will be larger than the first, and vice versa.
4. To solve for X, divide the product of the means by the known extreme. Cancel to simplify. Since the first and second terms form a ratio, common factors may be removed without altering the ratio; the first and third terms are actually numerators of equal fractions, so they can be divided by the same number without changing the proportion.

Example

If 100 g of a drug cost $1.80, how much will 25 g cost?

If the three quantities in the problem, namely 100 g, $1.80, and 25 g, are considered, it will be seen readily that 100 g bears the same relation to 25 g as $1.80 does to the unknown quantity to be calculated. In other words, the quantities and prices form equal ratios. The following proportion can be made:

$$100 \text{ g}:25 \text{ g}::\$1.80:X$$

There are three known terms in the statement and X, the unknown term. Arithmetically, the product of the means must equal the product of the extremes. Therefore, if one of the extremes is unknown, it may be calculated by dividing the product of the means by the known extreme.

$$X = \frac{25 \text{ g} \times \$1.80}{100 \text{ g}} = \$0.45$$

The proportion is set down preferably as given above, but it may be stated in several other ways. These are given below merely to show their relationship to the original form. It may be stated as two equal ratios in equation form:

$$\frac{100 \text{ g}}{25 \text{ g}} = \frac{\$1.80}{X}$$

DIMENSIONAL ANALYSIS

The basis for dimensional analysis is the formation of relationships between quantities, multiplication and canceling units until only the units of the desired answer remain.

As in the example used previously, if 100 g of a drug cost $1.80, how much will 25 g cost?

Begin by collecting all of the information in the problem and identify all relationships with units and labels. In this problem, we know

$$\frac{\$1.80}{100 \text{ g drug}}, 25 \text{ g drug}$$

Write down the units you want for the answer.

$$= X \$$$

Identify a relationship from the problem that contains the units desired for the answer, forming the skeleton of the process.

$$\frac{\$1.80}{100 \text{ g drug}} ? = X \$$$

Complete the process by adding terms from the problem (or equivalents) necessary to cancel out units until only the units of the answer remain on the left side.

$$\frac{\$1.80}{100 \text{ g drug}} 25 \text{ g drug} = X \$$$

Solve mathematically.

$$X = \$0.45$$

Dimensional analysis can be used to solve most pharmaceutical problems, regardless of complexity, using a consistent procedure:

1. Collect all the information and relationships in the problem complete with units and labels.
2. Write down the units and label of the answer.
3. Select a starting point corresponding to the units and label of the answer in the numerator.
4. Complete the process using relationships in the problem and known conversions to cancel units.
5. Solve the problem mathematically.

More complex problems use the same basic procedure; eg, if 100 g of a drug cost $1.80, what would be the cost of the drug to prepare 4 f℥ of a solution containing 5 g of the drug per teaspoonful?

Collect all information and relationships:

$$\frac{\$1.80}{100 \text{ g drug}}, \frac{5 \text{ g drug}}{1 \text{ teasp}}, 4 \text{ f℥}.$$

Present Steps 2–4 together:

$$\frac{\$1.80}{100 \text{ g drug}} \times \frac{5 \text{ g drug}}{1 \text{ teasp}} \times \frac{1 \text{ teasp}}{5 \text{ mL}} \times \frac{29.6 \text{ mL}}{1 \text{ f℥}} \times 4 \text{ f℥} = x \$$$

(The 3rd and 4th terms are known definitions and equivalents needed to cancel units.)
Solve: $x = \$0.53$.

The problems in the remainder of this chapter will be illustrated using both problem-solving methods. Readers should evaluate both methods and select the one they feel most comfortable with.

Examples

1. Determine the dose for each ingredient contained in one dose of the following prescription.

℞
Solid A	....	300 mg
Solid B	....	150 mg
Solid C	....	200 mg

M ft capsules, D.T.D. No 12.

The directions to the pharmacist are to mix and send 12 capsules containing in the three solids in the amounts indicated. Thus, the dose of each ingredient is as stated in the prescription.

2. How much of each ingredient is used in compounding the following prescription?

℞
Drug E	....	7.2 g
Drug F	....	0.24 g
Drug G	....	1.2 g

M div capsules, No 24.

In this prescription the prescriber requests that 24 capsules be made from the three ingredients. The amounts of the ingredients requested are considerable, and drugs usually do not have doses of 7.2 g or 1.2 g, so division of the amounts by the number of doses (24) is required. The pharmacist should check a textbook or compendium to confirm the average adult dose.

$$\text{Drug E:} \frac{7.2 \text{ g}}{24 \text{ caps}} \times 1 \text{ cap} = 0.300 \text{ g}$$

$$\text{Drug F:} \frac{0.24 \text{ g}}{24 \text{ caps}} \times 1 \text{ cap} = 0.01 \text{ g}$$

$$\text{Drug G:} \frac{1.2 \text{ g}}{24 \text{ caps}} \times 1 \text{ cap} = 0.05 \text{ g}$$

3. A prescription calls for 10 units of a drug to be taken 3 times a day. How much will the patient have taken after 7 days?

$$10 \text{ units/dose} \times 3 \text{ doses/day} \times 7 \text{ days} = 210 \text{ units}$$

4. If 250 units of an antibiotic weigh 1 mg, how many units are in the 15 mg?

$$250 \text{ units/mg} \times 15 \text{ mg} = 3750 \text{ units}$$

5. If the dose of a drug is 0.5 mg/kg of body weight/24 hours, how many grams will a 33-lb infant receive per 24 hours and per week?

$$\frac{1 \text{ g}}{1000 \text{ mg}} \times \frac{0.5 \text{ mg}}{\text{kg} \times 24 \text{ hours}} \times \frac{1 \text{ kg}}{2.2 \text{ lb}} \times 33 \text{ lb} \times 24 \text{ hours} = 0.0075 \text{ g}$$

$$\frac{0.0075 \text{ g}}{\text{day}} \times \frac{7 \text{ days}}{\text{week}} \times 1 \text{ week} = 0.0525 \text{ g}$$

6. A patient is to receive 260 μg of a drug 4 times a day for 14 days. How many 1/250-gr tablets must be dispensed?

$$\frac{1 \text{ tab}}{\frac{1}{250} \text{ gr}} \times \frac{1 \text{ gr}}{64.8 \text{ mg}} \times \frac{1 \text{ mg}}{1000 \mu\text{g}} \times \frac{260 \mu\text{g}}{\text{dose}} \times \frac{4 \text{ doses}}{\text{day}} \times 14 \text{ days} = 56 \text{ tabs}$$

7. An antibiotic is available as an injection containing 10 mg antibiotic/mL. How many mL are needed for an infant weighing 8 kg, the dose being 1.4 mg/kg of body weight?

$$\frac{1 \text{ mL}}{10 \text{ mg}} \times \frac{1.4 \text{ mg}}{\text{kg}} \times 8 \text{ kg} = 1.12 \text{ mL}$$

8. A preparation for coughs contains 1.5 g of an expectorant per 100 mL. How many gr of the expectorant are there in a teaspoonful?

$$1 \text{ teaspoonful} = 5\text{mL}$$

$$\frac{15.4 \text{ gr}}{1 \text{ g}} \times \frac{1.5 \text{ g}}{100 \text{ mL}} \times \frac{5 \text{ mL}}{1 \text{ teasp}} \times 1 \text{ teasp} = 1.16 \text{ gr}$$

Problems

1. Calculate the dose for each ingredient in the following prescription.

℞
Chemical J .... 10 mg
Chemical K .... 50 mg
Chemical L .... 300 g

<center>M ft capsules, D.T.D. No 14.</center>

2. Calculate the dose of each ingredient in the following prescription.

℞
Drug Q .... 10.5 g
Drug R .... 6.3 g

<center>Make 21 doses.</center>

3. An 8 fl oz prescription contains 6 fl dr of a tincture. If 1 teaspoonful 4 times a day is prescribed, how much tincture does the patient take per dose and how much is taken daily?

4. How many 0.3-mL doses are contained in 15 mL of a solution?

5. If 1 mg of a hormone equals 22.5 units, how many mg are required to obtain 1 unit?

6. If a bottle contains 80 units of a drug/mL, how many mL must the patient take to get a 60-unit dose? If the bottle contains 10 mL total volume of the drug solution, how many days' supply will patients have if they use 60 units a day?

7. A 10-mL ampul contains a 2.5% solution of a drug. How many mL are needed to give a dose of 150 mg?

8. The dose of an antibiotic is 75 mg for a child. How much of a flavored suspension containing 125 mg antibiotic/5 mL must be given to the child per dose?

9. How many gr of a drug are there in each teaspoonful of a syrup that contains 0.5% of the drug?

REDUCING AND ENLARGING FORMULAS

Determine the total weight or volume of ingredients and convert, if necessary, to the system of the quantities desired. The quantities in the original and new formulas will have the same ratio.

Examples

1. The formula for a syrup is

Drug M .... 140 g
Sucrose .... 450 g
Purified Water, qs to make .... 1000 mL

a. Find the quantities required for 100 mL.

$$\text{Drug M:}\frac{140\text{g}}{1000 \text{ mL}} \times 100 \text{ mL} = 14.0 \text{ g}$$

$$\text{Sucrose: }\frac{450 \text{ g}}{1000 \text{ mL}} \times 100 \text{ mL} = 45.0 \text{ g}$$

<center>Purified Water: to make 100 mL</center>

b. What quantities are required to compound 60 mL of the syrup?

$$\text{Drug M: }\frac{140 \text{ g}}{1000 \text{ mL}} \times 60 \text{ mL} = 8.40 \text{ g}$$

$$\text{Sucrose: }\frac{450 \text{ g}}{1000 \text{ mL}} \times 60 \text{ mL} = 27.0 \text{ g}$$

<center>Purified Water: to make 60 ml</center>

2. Calculate the amounts needed for 100 g of antiseptic powder as follows:

Solid A .... 2 g
Solid B .... 1 g
Solid C .... 7 g
Solid D .... 25 g
Solid E .... <u>115 g</u>

<div align="right">150 g</div>

Solid A	2 g × 0.667 =	1.33 g
Solid B	1 g × 0.667 =	0.667 g
Solid C	7 g × 0.667 =	4.67 g
Solid D	25 g × 0.667 =	16.7 g
Solid E	115 g × 0.667 =	<u>76.7 g</u>
		100.067 g

3. Prescriptions, where the instruction to the pharmacist calls for making a certain number of doses of an ingredient or mixture of several ingredients, are a type of formula enlargement. The expression usually used is DTD, which means let such doses be given (see Table 98-1). Occasionally the prescriber will not use this expression, but inspection of amounts of the ingredients indicates that this is what is desired. For example,

℞
Solid H .... 50 mg
Solid K .... 150 mg
Liquid N .... 0.2 mL

<center>M ft capsules, D.T.D. No 24.</center>

The pharmacist checked the individual doses of the ingredients and found them to be slightly below the average adult dose, confirming that the prescriber wanted the quantities listed to be multiplied by 24.

INGREDIENTS	AMOUNTS	MULTIPLIER	NEW AMOUNTS
Solid H	50 mg	×24	1200 mg or 1.2 g
Solid K	150 mg	×24	3600 mg or 3.6 g
Liquid N	0.2 mL	×24	4.8 mL

Problems

1. The formula for a liquid preparation is

Liquid C .... 35 mL
Solid B .... 9 g
Liquid R .... 2.5 mL
Liquid P .... 20 mL
Purified Water, sufficient to make .... 100 mL

Calculate the quantities of the ingredients to make 2.5 L.

2. The formula for an ointment is

℞
Solid G 1
Liquid D 30
Solid M 3
Ointment base, sufficient to make 1

Calculate quantities of the ingredients for 2 lb.

3. How much of each of the three solids and how much purified water are needed to properly compound the following prescription order?

℞
Solid N .... 0.1 mg
Solid Q .... 2.5 mg
Solid R .... 150.0 mg
Purified Water, qs to make .... 5 mL

<center>M ft solution, D.T.D. No 48.</center>

4. How much of each ingredient is required to compound 90 mL of the following product?

Solid S	7.5 g
Solid T	25 g
Oil C	350 mL
Alcohol	250 mL
Purified Water, qs, to make	1000 mL

PERCENTAGE

Percent, written as %, means per hundred. Fifteen percent is written 15% and means 15/100, 0.15, or 15 parts in a total of 100 parts. Percent is a type of ratio and has no units. Thus, 10% of 1500 tables is 10/100 × 1500 tablets = 150 tablets.

To change percent to a fraction, the percent number becomes the numerator and 100 is the denominator. To change a fraction to percent, put the fraction in a form having 100 as its denominator; multiply by 100 so that the numerator becomes the percent.

$$\tfrac{1}{2} = \frac{50}{100}; \quad \frac{50}{100} \times 100 = 50\%$$

$$\tfrac{1}{8} = \frac{12.5}{100}; \quad \frac{12.5}{100} \times 100 = 12.5\%$$

Calculations involving percentages are encountered continually by pharmacists. They must be familiar not only with the arithmetical principles, but also with certain compendial interpretations of the different type percentages involving solutions and mixtures.

The USP states

Percentage concentrations of solutions are expressed as follows:
Percent weight in weight—(w/w) expresses the number of g of a constituent in 100 g of solution.
Percent weight in volume—(w/v) expresses the number of g of a constituent in 100 mL of solution, and is used regardless of whether water or another liquid is the solvent.
Percent volume in volume—(v/v) expresses the number of mL of a constituent in 100 mL of solution.
The term percent used without qualification means, for mixtures of solids, percent weight in weight; for solutions or suspensions of solids in liquids, percent weight in volume; for solutions of liquids in liquids, percent volume in volume; and for solutions of gases in liquids, percent weight in volume. For example, a 1 percent solution is prepared by dissolving 1 g of a solid or 1 mL of a liquid in sufficient of the solvent to make 100 mL of the solution.

Ratio Strength

Ratio is another manner of expressing strength. Such phrases as "1 in 10" are understood to mean that 1 part by volume of a liquid is to be diluted with, or 1 part by weight of a solid dissolved in sufficient of the solution to make the finished solution 10 parts by volume. For example, a 1:10 solution means 1 mL of a liquid or 1 g of a solid dissolved in sufficient solvent to make 10 mL of solution. It can be converted to percent by

$$1 \text{ g}:10 \text{ mL}::X \text{ g}:100 \text{ mL}$$

$$X = 10 \text{ g in 100 mL of solution which is } 10\%$$

or

$$\frac{1 \text{ g}}{10 \text{ mL}} \times 100 \text{ mL} = 10 \text{ g}$$

$$\frac{10 \text{ g}}{100 \text{ mL}} = 10\%$$

The expression "parts per thousand" (eg, 1:5000) always means parts weight in volume when dealing with solutions of solids in liquids and is similar to the above expression. A 1:5000 solution means 1 g of solute in sufficient solvent to make 5000 mL of solution. This can be converted to percent by

$$1 \text{ g}:5000 \text{ mL}::X \text{ g}:100 \text{ mL}$$

$$X = 0.02 \text{ g in 100 mL solution which is } 0.02\%$$

or

$$\frac{1 \text{ g}}{5000 \text{ mL}} \times 100 \text{ mL} = 0.02 \text{ g}$$

$$\frac{0.02 \text{ g}}{100 \text{ mL}} = 0.02\%$$

The expression "trituration" has two different meanings in pharmacy. One refers to the process of particle-size reduction, commonly by grinding or rubbing in a mortar with the aid of a pestle. The other meaning refers to a dilution of a potent powdered drug with a suitable powdered diluent in a definite proportion by weight. It is the second meaning that is used in this chapter.

When pharmacists refer to a "1 in 10 trituration" they mean a mixture of solids composed of 1 g of drug plus sufficient diluent (another solid) to make 10 g of mixture of dilution. In this case the "1 in 10 trituration" is actually a solid dilution of a drug with an inert solid. The strength of a trituration may also be stated as percent w/w.

Thus, the term trituration has come to mean a solid dilution of a potent drug with a chemically and physiologically inert solid.

The meanings implied by the USP statements in the section on percentage are illustrated below with a few examples of the three types of percentages.

Weight-in-Volume Percentages

This is the type of percent problem most often encountered on prescriptions. The volume occupied by the solute and the volume of the solvent are not known because sufficient solvent is added to make a given or known final volume.

Examples

1. Prepare 1 f℥ of a 10% solution.

Since this is a solution of a solid in a liquid, this is a w/v solution.

$$\frac{10 \text{ g}}{100 \text{ mL}} \times \frac{29.6 \text{ mL}}{\text{f℥}} \times 1 \text{ f℥} = 2.96 \text{ g}$$

2.96 g is dissolved in sufficient purified water to make 29.6 mL of solution.

2. How much of a drug is required to compound 4 f℥ of a 3% solution in alcohol?

$$\frac{3 \text{ g}}{100 \text{ mL}} \times \frac{29.6 \text{ mL}}{\text{f℥}} \times 4 \text{ f℥} = 3.55 \text{ g}$$

3. How much 0.9% solution of sodium chloride can be made from ½ ℥ of NaCl?

$$\frac{100 \text{ mL}}{0.9 \text{ g}} \times \frac{31.1 \text{ g}}{1 \text{ ℥}} \times \frac{1}{2} \text{ ℥} = 1730 \text{ mL}$$

4. How many grams of a drug are required to make 120 mL of a 25% solution?

$$\frac{25 \text{ g}}{100 \text{ mL}} \times 120 \text{ mL} = 30 \text{ g drug}$$

5. How would you prepare 480 mL of a 1 in 750 solution of an antiseptic?

Remember: percent *w/v* is indicated.
1 in 750 means 1 g of the antiseptic dissolved in sufficient solvent to make 750 mL solution.
By ratio and proportion,

$$1 \text{ g}:750 \text{ mL}::U \text{ g}:480 \text{ mL}$$

$$U = 1 \text{ g} \times 480 \text{ mL}/750 \text{ mL} = 0.64 \text{ g antiseptic needed}$$

Dissolve 0.64 g of antiseptic in sufficient solvent to make 480 mL solution.

6. How much of a substance is needed to prepare 1 L of a 1:10,000 solution?

The ratio 1:10,000 means 1 g of a substance in 10,000 mL of solution.

$$1 \text{ L} = 1000 \text{ mL}$$

By ratio and proportion,

$$1 \text{ g}:10,000 \text{ mL}::V \text{ g}:1000 \text{ mL}$$
$$V = 1 \text{ g} \times 1000 \text{ mL}/10,000 \text{ mL} = 0.1 \text{ g substance needed}$$

7. How would you prepare 120 mL of 0.25% solution of neomycin sulfate? The source of neomycin sulfate is a solution which contains 1 g neomycin sulfate/10 mL.

$$\frac{10 \text{ mL stock soln}}{1 \text{ g drug}} \times \frac{0.25 \text{ g drug}}{100 \text{ vol soln}} \times 120 \text{ mL soln} = 3 \text{ mL stock soln}$$

Add sufficient purified water to 3 mL of stock solution to make 120 mL.

Problems

1. How would you make 3 fl oz of a 12.5% solution?
2. How many liters of a 4% solution can be made from 4 oz of a solid?
3. How many liters of an 8% solution can be made from 500 g of a solid?
4. How many grams of a drug are needed to make 4 L of a 1 in 500 solution?

Weight-in-Weight Percentages

Density must be considered in some of these problems. If a weight-in-weight solution is requested on a prescription, both the solute and solvent must be weighed, or the solute and the solvent may be measured if their densities are taken into consideration in determining the volumes. Since the solutions are made to a given weight, a given volume is not always obtainable.

Examples

1. What weights of solute and solvent are required to make 2 ʒ of a 3% *w/w* solution of a drug in 90% alcohol?

$$\frac{3 \text{ g solute}}{100 \text{ g soln}} \times \frac{31.1 \text{ g soln}}{1 \text{ ʒ soln}} \times 2 \text{ ʒ soln} = 1.87 \text{ g solute}$$

$$\frac{31.1 \text{ g soln}}{1 \text{ ʒ soln}} \times 2 \text{ ʒ soln} = 62.2 \text{ g soln}$$

$$62.2 \text{ g soln} - 1.87 \text{ g solute} = 60.3 \text{ g solvent}$$

2. The solubility of boric acid is 1 g in 18 mL of water at 25°C. What is the percentage strength, *w/w*, of a saturated solution?

1 g of boric acid + 18 mL of water make a saturated solution, 18 mL of water weighs 18 g; hence, the weight of solution is 19 g. The amount of boric acid present is 1 g in 19 g of solution; therefore, the following proportion can be set up:

$$1 \text{ g}:19 \text{ g}::X \text{ g}:100 \text{ g}$$

$$X = 1 \text{ g} \times 100 \text{ g}/19 \text{ g} = 5.26 \text{ g } 5.26 \text{ g}/100 \text{ g or } 5.26\%$$

3. How many grams of a chemical are needed to prepare 200 g of a 10% *w/w* solution?

10% *w/w* means 10 g of solute in 100 g total solution. If 100 g solution contains 10 g of solute, there are 90 g of solvent (100 g of solution – 10 g of solute = 90 g of solvent). The following proportion may be set up:

$$10 \text{ g}:100 \text{ g}::M \text{ g}:200 \text{ g}$$

$$M = 10 \text{ g} \times 200 \text{ g}/100 \text{ g} = 20 \text{ g solute needed}$$

4. How would one make a 2% *w/w* solution of a drug in 240 mL of alcohol? The density of alcohol is 0.816 g/mL.

a. First, convert 240 mL to weight. Remember: alcohol is the solvent and it has a density different from that of water.

$$\text{Density} = \text{Weight/Volume}$$

$$\text{Weight} = \text{Density} \times \text{Volume}$$

$$\text{Weight} = 0.816 \text{ g/mL} \times 240 \text{ mL} = 195.8 \text{ g (196 g)}$$

b. 2% *w/w* means 2 g solute in 100 g solution. In this problem the final weight of solution is not known; 240 mL (196 g) of alcohol represents the solvent only. The solvent is 98% *w/w* of the total solution, so the following proportion may be set up:

$$2 \text{ g}:98 \text{ g}::N \text{ g}:196 \text{ g}$$

$$N = 2 \text{ g} \times 196 \text{ g}/98 \text{ g} = 4.00 \text{ g}$$

c. Dissolve 4.00 g of the drug in 240 mL alcohol. The resulting solution will be 2% *w/w* and have a volume slightly larger than 240 mL because of the volume displacement of the drug.

5. How much of a 5% *w/w* solution can be made from 28.4 g of a chemical?

$$\frac{100 \text{ g soln}}{5 \text{ g chem}} \times 28.4 \text{ g chem} = 568 \text{ g soln}$$

6. How many mL of a 70% *w/w* solution having a density of 1.2 g/mL will be needed to prepare 600 mL of a 10% *w/v* solution?

a. $10 \text{ g}:100 \text{ mL}::Z \text{ g}:600 \text{ mL}$

$$Z = 60 \text{ g of drug needed}$$

b. $70 \text{ g}:100 \text{ g}::60 \text{ g}:T \text{ g}.$

$$T = 85.7 \text{ g of } 70\% \text{ } w/w \text{ solution needed.}$$

c. Volume = Weight/Density = 85.7 g/1.2 g/mL = 71.4 mL of the 70% *w/w* solution needed.

Compounding problems involving solid preparations (such as mixtures of powder) and semisolid preparations (such as ointments, creams, and suppositories) are also percent *w/w*. The following is an example of this.

1. How much drug is required to make 2 ʒ of a 10% ointment?

$$\frac{10 \text{ g drug}}{100 \text{ g oint}} \times \frac{31.1 \text{ g oint}}{1 \text{ ʒ oint}} \times 2 \text{ ʒ} = 6.22 \text{ g drug}$$

The same procedure could be used for such mixtures as powders and suppository masses. Instead of using units in the various measuring systems, quantities can be indicated "by parts." The term "parts" then can mean any unit in any measuring system, as long as the units are kept constant.

2. How many grams of each of the following three ingredients are required to make 30 g of the product?

℞
Solid A . . . . . . : . . . . 0.5 part
Powder B . . . . 3.0 parts
Powder C, qs to . . . . 30.0 parts

Since the product is a mixture of powders, percent *w/w* is indicated. In the above prescription order the total product is 30 parts because

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Powder C is used to "qs to" or "make up to" 30 parts. Therefore, 0.5 g of Powder A and 3.0 g of Powder B are needed.

Powder A	0.5 g
Powder B	+ 3.0 g
	3.5 g

Total product	30.0 g
	− 3.5 g
	26.5 g Powder C needed

3.

℞
Solid D	3.0 part
Solid E	6.0 parts
Ointment Base Q	30.0 parts

How much of each of the following ingredients is needed to make 60 g of the ointment? ?

Solid D	3.0 part
Solid E	6.0 parts
Ointment Base Q	30.0 parts
	39.0 parts total

Since a total of 60 g is needed, the following proportions can be made:

39 Parts total:60 g total needed::3.0 parts Solid D:X g

X = 60 g × 3.0 parts/39 parts = 4.62 g Solid D needed

39 Parts:60 g::6.0 parts:Y g

Y = 60 g × 6.0 parts/39 parts = 9.23 g Solid E needed

4.62 g Solid D	60.00 g
+ 9.23 g Solid E	−13.85 g Total
13.85 g	46.15 g Base Q needed

The amount of ointment base needed can also be calculated by the above ratio and proportion method.

4. What is the percent strength of a salt solution obtained by diluting 100 g of a 5% solution to 200 g?

Assign the 5% solution as Soln 1
Assign the final solution as Soln 2

$$\frac{5\text{ g drug}}{100\text{ g Soln 1}} \times \frac{400\text{ g Soln 1}}{200\text{ g Soln 2}} \times 100\text{ g Soln 2} = 2.5\text{ g drug}$$

$$\frac{2.5\text{ g drug}}{100\text{ g Soln 2}} = 2.5\%\ w/w$$

Problems

1. How much of the drug and solvent are needed to compound the following prescription?

℞
Compound A	6% w/w
Solvent, qs to make	4 ℨ

2. How many grams of solute are needed to prepare 240 g of a 12% w/w solution?

3. How many kg of a 20% w/w solution can be made from 1 kg of the solute?

4. How would you prepare, using 120 mL of glycerin (density, 1.25 g/mL), a solution that is 3% w/w with respect to a drug?

5. How much of each substance is needed to prepare a total of 24 g of the following suppository mass?

Compound K	0.3 g
Solid H	0.15 g
Suppository base, qs to make	2.0 g

6. How would one prepare 500 mL of a 15% w/w aqueous solution?

7. How much of each of the ingredients is required to make 1 kg of the following mixture?

Powder P	1 part
Powder Q	8 parts
Powder R	12 parts
Powder S	15 parts
	36 parts

8. How much of each ingredient is required to prepare the following ointment?

℞
Coal Tar Solution	10%
Hydrophilic Ointment, qs to make	30 g

Volume-in-Volume Percentages

A direct calculation of percentage from the total volume is made. Volumes, unlike weights, may not be additive. However, this does not present a problem because the final solution is up to the desired volume with the diluent.

Examples

1. How many minims of a liquid is needed to make 6 fl oz of a hand lotion containing 0.5% v/v of the liquid?

$$\frac{16.2\text{ ♏ liq}}{1\text{ mL liq}} \times \frac{0.5\text{ mL liq}}{100\text{ mL lotion}} \times \frac{29.6\text{ mL lotion}}{1\text{ f℥ lotion}} \times 6\text{ f℥ lotion} = 14.4\text{ ♏ liq}$$

Add sufficient lotion to 14.4 ♏= of the liquid to make 6 f℥ of the product.

2. How much 90% alcohol is required to compound 500 mL of a 10% alcohol mixture? In v/v mixtures, percentage is directly proportional to volume.

a. Since alcohol, a liquid, is mixed with water, percent v/v is indicated. Assume no shrinkage.

b. 500 mL of the 10% solution contains the following amount of alcohol:

10 mL:100 mL::X mL:500 mL

X = 10 mL × 500 mL/100 mL = 50 mL alcohol

c. 90% alcohol contains 90 mL of alcohol in 100 mL of solution. 50 mL of pure alcohol is needed; therefore, the following proportion may be set up:

90 mL:100 mL::50 mL:Y mL

Y = 100 mL × 50 mL/90 mL = 55.5 mL of 90% alcohol needed

Problems

1. How many minims of a liquid are needed to make 4 f℥ of a 12.5% v/v solution?

2. What volume of 50% v/v alcohol could be prepared from 1 L of 95% v/v alcohol?

3. What is the percentage strength, weight in weight, of a liquid made by dissolving 16 g of a salt in 30 mL of water?

4. How much drug will be required to prepare 1 fl oz of a 2.5% solution?

5. What is the percentage, weight in weight, of sugar in a syrup made by dissolving 5 kg of sugar in 8 kg of water?

6. How many grams of a drug are required to prepare 120 mL of a 12.5% aqueous solution?

7. How much drug is needed to compound a liter of a 1:2500 aqueous solution?

8. A solution contains 37% of active ingredient. How much of this solution is needed to prepare 480 mL of an aqueous solution containing 2.5% of the active ingredient?

9. How much of a drug is required to make 2 qt of a 1:1200 solution?

STOCK SOLUTIONS

To facilitate the dispensing of certain soluble substances, the pharmacist frequently prepares or purchases solutions of high concentration. Portions of these concentrated solutions are diluted to give required solutions of lesser strength. These concentrated solutions are known as *stock solutions*. This procedure is satisfactory if the substances are stable in solution or if the solutions are to be used before they decompose.

In the case of potent substances, a properly prepared stock solution permits the pharmacist to obtain accurately a quantity of solid that might otherwise be difficult to weigh. In the case of frequently prescribed salt solutions, a stock solution readily provides the required amount of salt without the necessity of weighing and dissolving it every time.

Stock solutions may be of various concentrations depending on the requirements for use. The stock solutions should be labeled properly and fractional parts needed to make various strengths also may be listed as a further convenience.

There is a type of compounding and dispensing problem that involves the concept of stock solutions. This involves the patient diluting a dose from the prescription order to a given volume to obtain a solution of desired concentration.

For example, how many grams of a salt are required to make 90 mL of a stock solution, 5 mL of which makes a 1:3000 solution when diluted to 500 mL?

a. Determine how many grams are in 500 mL of a 1:3000 solution.

$$1 \text{ g}:3000 \text{ mL}::X \text{ g}:500 \text{ mL}$$

$$X = 1 \text{ g} \times 500 \text{ mL}/3000 \text{ mL} = 0.167 \text{ g}$$

salt in 500 mL of 1:3000 solution

b. The 0.167 g in the dilute solution came from the 5 mL of the original stock solution (prescription order). The following proportion can be used.

$$0.167 \text{ g}:5 \text{ mL}::Y \text{ g}:90 \text{ mL}$$

$$Y = 0.167 \text{ g} \times 90 \text{ mL}/5 \text{ mL} = 3.01 \text{ g}$$

salt needed to make the original 90 mL of stock solution

Alternately,

Assign stock solution as Soln 1
Assign final dilution as Soln 2

$$\frac{1 \text{ g salt}}{3000 \text{ mL Soln 2}} \times \frac{500 \text{ mL Soln 2}}{5 \text{ mL Soln 1}} \times 90 \text{ mL Soln 1} = 3.0 \text{ g salt}$$

Problems

1. How much of a drug is needed to compound 120 mL of a prescription order such that when 1 teaspoonful of the solution is diluted to 1 qt, a 1:750 solution results?

2. How many grams of a drug are needed to make 240 mL of a solution of such strength that when 5 mL is diluted to 2 qt, a 1:2500 solution results?

3. An ampul of solution of an anti-inflammatory drug contains 4 mg of drug/mL. What volume of the solution is needed to prepare a liter of solution that contains 2 μg of the drug/mL?

PARTS PER MILLION

An expression that is occasionally used in compounding prescriptions is *parts per million* (ppm). This is another way of expressing concentration, particularly concentrations of very dilute preparations. A 1% solution may be expressed as 1 part/100; a 0.1% solution is 0.1 part/100 or 1 part/1000. A 1 ppm

solution contains 1 part of solute/1 million parts of solution; 5 ppm is 5 parts solute/1 million parts solution, and so on. Remember that the two parts must have the same units, except in the metric system where 1 g = 1 mL of water.

Sodium fluoride is a drug that may be prescribed by a dentist as a preventative for tooth decay in children. It is used only in very dilute solutions due to the drug's toxicity and because only minute quantities are needed. For example, how much sodium fluoride would be needed to prepare the following prescription?

℞
Sod Fluoride, qs
Purified water, qs 60 mL
Make soln such that when 1 f℥ is diluted to 1 glassful of water a 2 ppm
    soln results.
Sig: 1 f℥ in a glassful of water a day.

The mathematics to solve this compounding problem are easy once the steps for calculating the answer are outlined. This problem should be worked "backward."

a. The amount of NaF needed is not known.
b. One glassful of water has a volume of 240 mL. The concentration of NaF in 240 mL is 2 ppm.
c. The NaF solution poured into the glass came from a teaspoonful dose (1 ℥), which is equal to 5 mL.
d. The 5-mL dose came from the prescription order bottle containing a NaF solution.

Now, inserting numbers

a. 240 mL contains 2 ppm NaF.

$$2 \text{ g}:1,000,000 \text{ mL}::X \text{ g}:240 \text{ mL}$$

$$X = 2 \text{ g} \times 240 \text{ mL}/1,000,000 \text{ mL} = 0.00048 \text{ g}$$

b. The 0.00048 g of NaF in the glass came from the teaspoonful dose; therefore, the teaspoonful (5 mL) contained 0.00048 g of NaF.
c. The 5 mL came out of the original prescription order bottle (60 mL).

$$5 \text{ mL}:0.00048 \text{ g}::60 \text{ mL}:Y \text{ g}$$

$$Y = 0.00048 \text{ g} \times 60 \text{ mL}/5\text{mL} = 0.00576 \text{ g}$$

The pharmacist would weigh out 5.76 mg (actually, one would weigh out a larger quantity and take an aliquot part) and qs to 60 mL.

Another variation of this problem is the prescriber requesting the concentration in terms of fluoride ion (F⁻). In this case the atomic weight of F⁻ and molecular weight of NaF are used in the calculation. If the request called for 2 ppm fluoride, the initial calculations would be the same as above, and an additional step would be added at the end. The 5.76 mg would now represent the weight of fluoride ion needed. This must be converted to weight of NaF. The molecular weight of NaF is 42 and the atomic weight of fluorine is 19. The following proportion can be set up.

$$5.76 \text{ mg}:19::Z \text{ mg}:42$$

$$Z = 5.76 \text{ mg} \times 42/19 = 12.7 \text{ mg}$$

Problems

1. How many mg of NaF are needed in the following prescription?

℞
Sodium Fluoride
Purified water, qs to 90 mL
M ft solution such that when 1 ℥ is dil to 1 glassful of water a 3 ppm NaF
    soln results.

DILUTION AND CONCENTRATION

Stock solutions can be diluted to make a product that has a lower concentration; also mixtures of powders or semisolids (eg, ointments) can be diluted to give a product of lower concentration of the drug(s). The diluent is an inert solid or semisolid or base that does not contain any active ingredients.

Mixtures also may be concentrated by adding pure drug or mixing with a product containing a higher concentration of the drug. For example, how much of a diluent must be added to 50 g of a 10% ointment to make it a 5% ointment?

1. How many grams of active ingredient are in 50 g of 10% ointment?

$$10 \text{ g}:100 \text{ g}::V \text{ g}:50 \text{ g}$$

$$V = 10 \text{ g} \times 50 \text{ g}/100 \text{ g} = 5 \text{ g}$$

2. How many grams of a 5% ointment can be made from 5 g of active ingredient?

Assign the 10% ointment as Oint 1
Assign the 5% ointment as Oint 2

$$\frac{100 \text{ g Oint 2}}{5 \text{ g drug}} \times \frac{10 \text{ g drug}}{100 \text{ g Oint1}} \times 50 \text{ g Oint 1} = 100 \text{ g Oint 2}$$

3. How many grams of base must be added to the 50 g of the original 10% ointment?

100 g	5% ointment
− 50 g	10% base
50 g	base

The term *trituration* was used previously to mean a dilute powder mixture of a drug. It is often necessary to dilute this mixture further to obtain the required amount of drug.

1. How much of a 1 in 10 trituration of a potent drug contains 200 mg of the drug?

A 1 in 10 trituration means 1 g of drug in 10 g of mixture or 1 g of drug plus 9 g diluent. *Remember*: mixtures of solids are present *w/w*. The following proportion can be made:

$$1 \text{ g}:10 \text{ g}::0.2 \text{ g}:T \text{ g}$$

$$T = 10 \text{ g} \times 0.2 \text{ g}/1 \text{ g} = 2 \text{ g}$$

2. How much diluent must be added to 10 g of a 1:100 trituration to make a mixture that contains 1 mg of drug in each 10 g of the final mixture?

a. Determine the amount of drug in 10 g of trituration.

$$1 \text{ g}:100 \text{ g}::M \text{ g}:10 \text{ g}$$

$$M = 1 \text{ g} \times 10 \text{ g}/100 \text{ g} = 0.1 \text{ g}$$

b. Determine the amount of mixture that can be made from 0.1 g (100 mg) of drug.

$$1 \text{ mg}:10 \text{ g}::100 \text{ mg}:N \text{ g}$$

$$N = 10 \text{ g} \times 100 \text{ mg}/1 \text{ mg} = 1000 \text{ g}$$

c. Determine the amount of diluent needed.

1000 g	total mixture
− 10 g	trituration
990 g	diluent

Problems

1. The following prescription order was received in a pharmacy. If the only R cream available is a 10% concentration, how much of the 10% cream and how much diluent are required to compound the prescription?

℞
R Cream 3% .... 30 g

2. How many grams of a 1:100 trituration contain 100 μg of the active ingredient?

3. How many grams of a 1:1000 dilution can be made from 1 g of a 1:25 trituration?

MIXING DIFFERENT STRENGTHS

Rules

1. The sum of the products obtained by multiplying a series of quantities by their respective concentrations equals the product obtained by multiplying a concentration by the sum of the quantities. For example, the sum of the products—obtained by multiplying the individual weights or volumes of a series of preparations by the concentration of a given ingredient contained in each preparation—is equal to the product obtained by multiplying the total weight of the series of preparations by the percentage of the given ingredient resulting from a homogeneous mixture of the same series of preparations.

2. When mixing products of varying strengths, the units and type of percent (*w/w, w/v, v/v*) must be kept constant.

Examples

1. What is the percent of alcohol in a mixture made by mixing 5 L of 25%, 1 L of 50%, and 1 L of 95% alcohol?

a. Determine the total amount of alcohol in the three solutions and the total amount of solution (1 L = 1000 mL). Assume additivity of volumes on mixing.

$$\frac{25 \text{ mL alc}}{100 \text{ mL}} \times 5000 \text{ mL} = 1250 \text{ mL alc}$$

$$\frac{50 \text{ mL alc}}{100 \text{ mL}} \times 1000 \text{ mL} = 500 \text{ mL alc}$$

$$\frac{95 \text{ mL alc}}{100 \text{ mL}} \times \frac{1000 \text{ mL}}{7000 \text{ mL}} = 950 \text{ mL alc}$$

b. Determine the percent of alcohol in the mixture. There is a total of 2700 mL of alcohol in 7000 mL of total solution.

$$\frac{2700 \text{ mL alc}}{7000 \text{ mL}} \times 100 \text{ mL} = 38.6 \text{ mL alc}$$

$$\frac{38.6 \text{ mL alc}}{100 \text{ mL}} = 38.6\%$$

2. What is the strength of a mixture obtained by mixing 50 g of a 5%, 100 g of a 7.5% and 40 g of a 10% ointment?

a.

5% ×	50 g	=	2.5 g
7.5% ×	100 g	=	7.5 g
10 % ×	40 g	=	4.0 g
	190 g		14.0 g

b. There is a total of 14.0 g of active ingredient in 190 g of total mixture.

$$14 \text{ g}:190 \text{ g}::W \text{ g}:100 \text{ g}$$

$$W = 14 \text{ g} \times 100 \text{ g}/190 \text{ g} = 7.37 \text{ g}$$

Since there are 7.37 g of active ingredients in 100 g of the mixture, a 7.37% preparation results.

Problems

1. What percent of a drug is contained in a mixture of powder consisting of 0.5 kg, containing 0.038% of a drug, and 10 kg, containing 0.043% of a drug?

2. What is the strength of a mixture produced by combining the following lots of alcohol: 2 L of 95%, 2 L of 50%, and 7 L of 60%?

3. What is the percent of drug content in the following mixture: 2 kg of 3%, 300 g of 2.5%, and 500 g of 4.2% resin?

ALLIGATION ALTERNATE

Alligation is a rapid method of calculation that is useful to the pharmacist. The name is derived from the Latin *alligatio*, meaning the act of attaching, and it refers to lines drawn during calculation to bind quantities together. This method is used to find the proportions in which substances of different strengths or concentrations must be mixed to yield a mixture of desired strength or concentration. When the proportion is found, a calculation may be performed to find the exact amounts of the substances required.

Rules

1. The substance with a higher value than that required is the one with the lower amount.

2. The gain in value or amount of one substance balances the loss in value or amount of another substance.

Examples and Procedure

1. In what proportion must a preparation containing 10% of drug be mixed with one containing 15% of drug to produce a mixture of 12% drug strength?

The 10% drug is 2% too weak and the 15% is 3% too strong. Therefore, the excess in strength of three parts of the stronger can be calculated to just balance the deficiency of two parts of the weaker drug. Set up the problem in this manner:
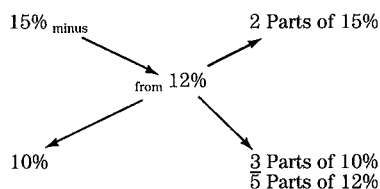
The desired percent or concentration is placed in the center, the lower percentage is placed on the left side below the center and the higher percentage is placed on the left side above the center. The figure obtained by subtracting 10% from 12% is placed opposite the 15% on the right side, and that obtained by subtracting the 12% from 15% is placed opposite the 10% figure on the right side.

Then, mixing 2 parts of 15% drug preparation with 3 parts of 10% drug preparation will produce a drug mixture of the desired 12% strength.

2. In what proportion must 30% alcohol and 95% alcohol be mixed to make 500 mL of 50% alcohol? Set up the problem in the following manner:
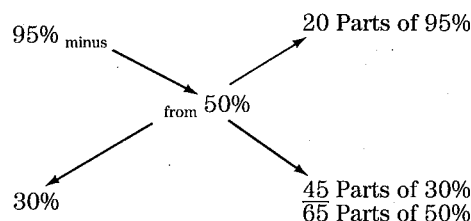
In a total of 65 parts, 20 parts of 95% alcohol + 45 parts of 30% alcohol are needed. Since the total parts is proportional to 500 mL, the following proportion can be made:

$$\frac{20 \text{ part (mL) } 95\%}{65 \text{ part (mL) } 50\%} \times 500 \text{ mL } 50\% = 154 \text{ mL } 95\%$$

Add sufficient 30% alcohol to make 500 mL.

3. How many grams of an ointment containing 0.18% of active ingredient must be mixed with 50 grams of an ointment containing 0.14% of active ingredient to make a product containing 0.15% of active ingredient?
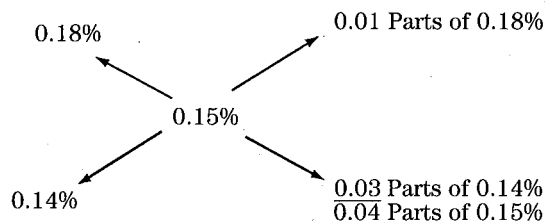
$$\frac{0.01 \text{ parts (g) } 0.18\%}{0.03 \text{ parts (g) } 0.14\%} \times 50 \text{ g } 0.14\% = 16.7 \text{ g } 0.18\%$$

4. Occasionally, it is necessary for a pharmacist to increase the strength of a product. For example, a prescription calls for 50 g of a 10% ointment. The pharmacist only has a 5% ointment and the pure ingredient available. How much of the 5% ointment and the pure ingredient are needed to compound the prescription?
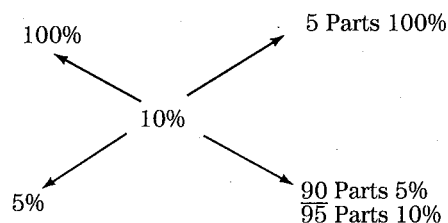
$$\frac{5 \text{ parts (g) } 100\%}{95 \text{ parts (g) } 10\%} \times 50 \text{ g } 10\% = 2.63 \text{ g } 100\%$$

$$\frac{90 \text{ parts (g) } 5\%}{95 \text{ parts (g) } 10\%} \times 50 \text{ g } 10\% = 47.4 \text{ g } 5\%$$

Problems

1. How much ointment containing 12% drug and how much ointment containing 16% drug must be used to make 1 kg of a product containing 12.5% drug?

2. In what proportion should 50% alcohol and purified water be mixed to make a 35% alcohol solution? (The purified water is 0% alcohol.)

Note: This problem may be solved by a method other than alligation as was shown above.

3. How many grams of 28% *w/w* ammonia water should be added to 500 g of 5% *w/w* ammonia water to produce a 10% *w/w* ammonia concentration?

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4. How many mL of 20% dextrose in water and how many mL of 50% dextrose in water are needed to make 1 L of 35% dextrose in water?

PROOF SPIRIT

For tax purposes, the US government calculates the strength of pure or absolute alcohol (herein referred to as C_2H_5OH) by means of *proof degrees*. This means that 100 proof spirit contains 50% (by volume) or 42.49% (by weight) of C_2H_5OH, and its specific gravity is 0.93426 at 60°F. Thus, 2 proof degrees equals 1% (by volume) of C_2H_5OH. One proof gallon is 1 gal of 50% (by volume) of C_2H_5OH at 15.56°C (60°F). In other words, a proof gallon is a gallon that contains ½ gal of C_2H_5OH. A proof gallon is 100 proof.

The term *10 degrees under proof* (10° up) signifies that 100 volumes of the spirit contains 90 volumes of proof spirit plus 10 volumes of water, and *30 degrees over proof* (30° op) indicates that 100 volumes diluted with water yields 130 volumes of proof spirit. To prepare proof spirit, 50 volumes of C_2H_5OH are mixed with 53.71 volumes of water to allow for the contraction that occurs to yield 100 volumes of product.

The terms *proof strength, proof gallon,* and *proof spirit* are used so that the tax is levied only on the actual quantity of C_2H_5OH contained in any mixture. Therefore, it is sometimes necessary for the pharmacist to convert alcohol purchased to proof strength to compute tax refunds or convert proof strengths to percent for compounding purposes.

A quantity of solution that contains ½ gal of C_2H_5OH is said to contain 1 proof gal. Proof gallons may be calculated by the following two equations:

$$\text{Proof gal} = \frac{\text{gal} \times v/v \text{ strength}}{50\% \, v/v}$$

$$\text{Proof gal} = \frac{\text{gal} \times \text{proof strength}}{100 \text{ proof}}$$

The second equation is the same as the first because proof strength is always twice the % *v/v* strength. With these equations, given any two variables the third can be calculated.

Examples

1. What is the taxable alcohol in 1 pt of Alcohol USP?

$$1 \text{ pt} = \tfrac{1}{8} \text{ gal (8 pt} = 1 \text{ gal)}$$

Alcohol USP is 95% *v/v*; therefore,

$$\text{Proof gal} = \frac{\text{gal} \times \% \text{ strength}}{50\%} = \frac{\tfrac{1}{8} \text{ gal} \times 95\%}{50\%} = 0.2375 \text{ proof gal}$$

2. How much Diluted Alcohol USP can be made from 1 qt of alcohol labeled ½ proof gallon?

Diluted Alcohol USP is 49% *v/v*; therefore,

$$\text{Proof gal} = \frac{\text{gal} \times \% \text{ strength}}{50\%}$$

$$\text{gal} = \frac{0.5 \text{ proof gal} \times 50\%}{49\%} = 0.510 \text{ gal}$$

Problems

1. How many proof gallons are there in 1 qt of a preparation that is labeled 75% *v/v* alcohol?
2. How many proof gallons are there in a pint of an elixir that contains 14% alcohol?
3. How much Diluted Alcohol USP can be made from 1 gal of 190 proof alcohol?

SATURATED SOLUTIONS

Occasionally, it is necessary for a pharmacist to make saturated solutions. Solubility in the USP/NF is expressed as the number of milliliters of a solvent that will dissolve 1 g of a solid; for example, 1 g dissolves in 0.5 mL of water. In other words, if 1 g of a solid is dissolved in 0.5 mL of water, a saturated solution results. An example will illustrate this.

How much of a drug is needed to make 120 mL of a saturated solution if 1 g of the drug dissolves in 7.5 mL of water?

Calculate the amount of drug that can be dissolved in 120 mL water.

$$X = 1 \text{ g} \times 120 \text{ mL/7.5 mL} = 16 \text{ g}$$

When 16 g of the drug are dissolved in 120 mL of water, a saturated solution results that has a volume greater than 120 mL because the solid will take up a certain volume. Only 120 mL would be dispensed.

What is the % *w/w* of the above solution?

$$120 \text{ g (mL) water} + 16 \text{ g drug} = 136 \text{ g of solution}$$

of total solution weight. There is 16 g of solute in 136 g of solution; therefore,

$$16 \text{ g:}136 \text{ g::}P \text{ g:}100 \text{ g}$$

$$P = 16 \text{ g} \times 100 \text{ g/136 g} = 11.8 \text{ g}$$

in 100 g of solution. Therefore, this is a 11.8% *w/w* solution.

Problems

1. What is the solubility of a chemical if a saturated aqueous solution is 0.5% *w/w*?
2. How many grams are needed to make 500 mL of a saturated solution if 1 g of the solute is soluble in 14 mL of solvent?

MILLIEQUIVALENTS

The quantities of electrolytes administered to patients are usually expressed by the term *milliequivalents* (mEq). The reason that weight units (mg, g) are not used is because the electrical activity of the ions, which in this instance is important, may be best expressed as mEq. (See Chapter 17 for additional discussion on electrolytic equilibria.)

A mEq is 1/1000 of an *equivalent* (Eq). An Eq is the weight of a substance that combines with or replaces one gram-atomic weight (g-at wt) of hydrogen. In pharmacy the terms equivalent and equivalent weight (Eq wt) have been used interchangeably. For problem solving it is convenient to identify the molar weight in terms of mg per mmol and the number of mEq per mmol as follows:

$$\text{Molecular weight} = \frac{\text{mg}}{\text{mmol}}$$

$$\text{mEq} = \frac{\text{mmol}}{\text{valence}}$$

For example, KCl has a molecular weight is 74.5; the above parameters would be 74.5 mg/mmol and 1 mEq/mmol.

Water of hydration contributes to the molecular weight (mol wt) of a compound but *not* to the valence, and the total mol wt is used to calculate mEq.

Examples

1. Calcium (Ca^{2+}) has a gram-atomic weight of 40.08. Determine the number of mEq/mmol. As the valence of the calcium ion in 2,

$$\frac{2 \text{ mEq}}{\text{mmol Ca}}$$

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2. A solution (100 mL) that contains 409.5 mg of NaCl/100 mL has how many mEq of Na^+ and Cl^-?

$$\text{Molecular wt} = 58.5 \text{ mg/mmol}$$

$$\text{There is } \frac{1 \text{ mEq } Cl^-}{\text{mmol NaCl}} \text{ and } \frac{1 \text{ mEq } Na^+}{\text{mmol NaCl}}$$

$$\frac{1 \text{ mEq } Cl^-}{\text{mmol NaCl}} \times \frac{1 \text{ mmol NaCl}}{58.5 \text{ mg NaCl}} \times \frac{409.5 \text{ mg NaCl}}{100 \text{ mL}} \times 100 \text{ mL}$$

$$= 7.0 \text{ mEq of } Na^+$$

3. A prescription order calls for a 500 mL solution of potassium chloride to be made so that it will contain 400 mEq of K^+. How many grams of KCl (mol wt: 74.5) are needed?

$$\frac{1 \text{ mEq}}{\text{mmol}} \text{ and } \frac{74.5 \text{ mg}}{\text{mmol}}$$

$$\frac{1 \text{ g KCl}}{1000 \text{ mg KCl}} \times \frac{74.5 \text{ mg KCl}}{\text{mmol KCl}} \times \frac{1 \text{ mmol KCl}}{\text{mEq } K^+}$$

$$\times 400 \text{ mEq } K^+ = 29.8 \text{ g KCl}$$

4. How many mEq of K^+ are in a 250-mg tablet of potassium phenoxymethyl penicillin (mol wt: 388.5; valence: 1)?

$$\frac{1 \text{ mEq } K^+}{\text{mmol Pen}} \text{ and } \frac{388.5 \text{ mg Pen}}{\text{mmol Pen}}$$

$$\frac{1 \text{ mEq } K^+}{\text{mmol Pen}} \times \frac{1 \text{ mmol Pen}}{388.5 \text{ mg Pen}} \times \frac{250 \text{ mg Pen}}{\text{tab}} \times 1 \text{ tab}$$

$$= 0.644 \text{ mEq } K^+$$

5. How many mEq of Mg are there in 10 mL of a 50% Magnesium Sulfate Injection? The mol wt of $MgSO_4 \cdot 7H_2O$ is 246.

$$\frac{2 \text{ mEq } Mg^{2+}}{\text{mmol drug}} \text{ and } \frac{246 \text{ mg drug}}{\text{mmol drug}}$$

$$\frac{2 \text{ mEq } Mg^{2+}}{\text{mmol drug}} \times \frac{1 \text{ mmol drug}}{246 \text{ mg drug}} \times \frac{1000 \text{ mg drug}}{\text{g drug}} \times \frac{50 \text{ g drug}}{100 \text{ mL}}$$

$$\times 10 \text{ mL} = 40.7 \text{ mEq } Mg^{2+}$$

6. A vial of Sodium Chloride Injection contains 3 mEq/mL. What is the percentage strength of this solution? The mol wt of NaCl is 58.5.

$$\frac{1 \text{ mEq}}{\text{mmol}} \text{ and } \frac{58.5 \text{ mg}}{\text{mmol}}$$

$$\frac{1 \text{ g}}{1000 \text{ mg}} \times \frac{58.5 \text{ mg}}{\text{mmol}} \times \frac{1 \text{ mmol}}{1 \text{ mEq}} \times \frac{3 \text{ mEq}}{\text{mL}} \times 100 \text{ mL}$$

$$= 17.6 \text{ g}$$

$$\frac{17.6 \text{ g}}{100 \text{ mL}} = 17.6\%$$

Problems

1. What is the mEq wt of ferrous ion (Fe^{2+}) which has a gram atomic weight of 55.85 g?

2. What is the mEq wt of sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$)?

3. How many mEq of Na are in 60 mL of an 5% solution of sodium saccharin (g mol wt: 241 g; valence:1)?

4. How many mEq of Ca^{2+} are there in a 600-mg calcium lactate pentahydrate (g mol wt: 308.30 g) tablet?

5. How many mEq of sodium are there in a 5 gr sodium bicarbonate tablet? The mol wt of $NaHCO_3$ is 84 and the valence is 1.

6. How many mEq of Na are there in 500 mL of ½ normal saline solution? Normal saline solution contains 9 g NaCl/L; mol wt NaCl is 58.5.

7. How much KCl is needed to make a pint of syrup that contains 10 mEq of K^+ in each tablespoonful? The mol wt of KCl is 74.5.

TEMPERATURE

Rules

The relationship of Centigrade (C) and Fahrenheit (F) degrees is

$$9(°C) = 5(°F) - 160$$

where °C is the number of degrees Centigrade, and °F is the number of degrees Fahrenheit.

Examples

1. Convert 77°F into °C.

$$9(°C) = 5(°F) - 160$$
$$9(°C) = 5(77) - 160$$
$$°C = \frac{385 - 160}{9} = 25°C$$

2. Convert 10°C into °F.

$$9(°C) = 5(°F) - 160$$
$$9(10) = 5(°F) - 160$$
$$\frac{90 + 160}{5} = °F = 50°F$$

Problems

Convert

a. 30°C into °F
b. 100°C into °F
c. 37°C into °F
d. 120°F into °C

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DENSITY

1. 816 g
2. 363 mL
3. 54.2 mL

LOGARITHMS

1. $V = -0.000084$ cm/sec or -8.4×10^{-5} cm/sec
2. $S = 65.6$ dynes/cm
3. $\dfrac{[\text{Un-ionized}]}{[\text{Ionized}]} = 7.94 \times 10^{-9}$

ADDITION

1. 2481.23 g or 2.48123 kg
2. 1157 g or 1.157 kg
3. 2 ʒ, 2 ꝫ, 3 ꝭ, 17½ gr
4. 1 ℔, 7 ꝫ, 4 ꝫ, 2 ꝭ, 4 gr
5. 2 79/90 gr
6. 4 gal, 2 qt, 11 fl oz

SUBTRACTION

1. 4105 mL or 4.105 L
2. 143½ gr
3. 7 fl oz, 2 fl dr
4. 3.71 g

MULTIPLICATION

1. 157 mL
2. 163 g
3. 825 mL
4. 2 gal, 1 qt, 4 fl oz, 4 fl dr
5. 375 mg

DIVISION

1. 769 capsules + 15 mg remainder
2. 160 capsules
3. 150 doses
4. 150 doses
5. 1396 capsules + 300 mg remainder

CONVERSIONS

1.

a. 422 mg
b. 19.4 mg
c. 109 g
d. 7780 mg
e. 99.4 g
f. 454 g
2.

a. 1 lb, 3 oz, 173 gr
b. 6.94 gr
c. 1 lb, 5 ꝫ, 5 ꝭ, 26 gr
d. 0.00154 gr
e. 2.2 lb
3.

a. 0.648 mg
b. 0.203 mg
c. 10.8 mg
d. 0.325 or 0.324 g
e. 1.299 or 1.296 g
4.

a. 12.3 mL
b. 11.1 mL
c. 237 mL
d. 473 mL
e. 0.309 mL
f. 0.00154 gr
g. 0.0772 gr

5.

a. 480 gr
b. 8 ꝫ
c. 437½ gr
d. 2880 gr
e. 4 ꝫ, 10 gr

DOSAGE CALCULATION

1. 1.5 mg
2. ½ gr
3. 18.7 mg
4. 280,000 units
5. 75 mg
6. 77.9 mg

PROBLEM-SOLVING METHODOLOGY

1. D.T.D. No. 14 means, send 14 such doses. Assuming the doses have been checked, they are for chemicals J, K, and L (10 mg, 50 mg, and 300 mg, respectively).
2. Drug Q: 0.5 g
    Drug R: 0.3 g
3. 7.6 ♏/dose: 30.4 ♏/day
4. 50 doses
5. 0.0444 mg
6. 0.75 mL contains 60 units: 13 1/3-day supply.
7. 6 mL
8. 3 mL
9. 0.386 gr

REDUCING AND ENLARGING

1. Liquid C 875 mL
    Solid B 225 g
    Liquid R 62.5 mL
    Liquid P 500 mL
2. Solid G 24 ꝫ or 3 ꝭ
    Liquid D 720 ♏= or 1 fꝫ, 4 fꝭ
    Solid M 72 ꝫ or 9ꝫ
3. Solid N 4.8 mg
    Solid Q 120 mg
    Solid R 7.2 g

Add sufficient purified water to make 240 mL solution.

4. Solid S 0.675 g
    Solid T 2.25 g
    Oil C 31.5 mL
    Alcohol 22.5 mL

PERCENTAGE
w/v Solutions

1. Dissolve 11.1 g in sufficient solvent to make 3 fꝫ.
2. 2.84 L
3. 6.25 L
4. 8 g

w/w Products

1. Compound A 115 gr or 1 ꝫ, 2 ꝭ, 15 gr
    Solvent 3 ꝫ, 365 gr
2. 28.8 g
3. 5 kg
4. Dissolve 4.64 g of drug in 120 mL (150 g) of glycerin.
5. Compound K 3.6 g
    Solid H 1.8 g
    Base 18.6 g
6. Dissolve 88.2 g of the solute in 500 mL of purified water. Dispense 500 mL
7. Powder P 27.8 g
    Powder Q 222.2 g
    Powder R 333.3 g
    Powder S 416.7 g
8. 3 g of coal tar solution; 27 g of hydrophilic ointment

PERCENT
(v/v, w/v, and w/w)

1. 240 ♏
2. 1900 mL

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3. 34.8% *w/w*
4. 11.4 gr
5. 38.5% *w/w*
6. 15 g
7. 0.4 g
8. 32.4 mL of a 37% solution
9. 1.58 g

STOCK SOLUTIONS

1. 30.3 g
2. 36.3 g
3. 0.5 mL

PARTS PER MILLION

1. 13 mg

DILUTION AND CONCENTRATION

1. 9 g of 10% cream and 21 g of diluent (base)
2. 0.01 g
3. 40 g

MIXING PRODUCTS OF DIFFERENT STRENGTHS

1. 0.0428%
2. 64.5%
3. 3.16%

ALLIGATION ALTERNATE

1. 875 g of 12% ointment and 125 g of 16% ointment

2. 35 parts of 50% alcohol and 15 parts of purified water
3. 139 g of 28% ammonia water
4. 500 mL each of the 20% and 50% solutions are needed

PROOF SPIRIT

1. 0.375 proof gal
2. 0.035 proof gal
3. 1.94 gal

SATURATED SOLUTIONS

1. 1 g in 199 mL
2. 35.7 g of solute

MILLIEQUIVALENTS

1. 27.925 mg/mEq
2. 89.3 mg/mEq
3. 12.5 mEq
4. 3.9 mEq
5. 3.86 mEq Na
6. 38.5 mEq Na
7. 23.5 g

TEMPERATURE

1.

a. 86°F
b. 212°F
c. 98.6°F
d. 48.9°C

JOINT APPENDIX 39

USP **NF**

1995

U. S. PHARMACOPEIA

NATIONAL FORMULARY

USP 23

NF 18

Continuous Revision of the USP and the NF

The need for continuously refining specifications and updating standards is a natural consequence of the introduction of new drugs and the accelerated growth of knowledge in the pharmaceutical sciences.

In order to keep the *United States Pharmacopeia* and the *National Formulary* abreast of these developments and to maintain the official standards accordingly, the main volume is revised regularly by *Supplements. Interim Revision Announcements* are issued between *Supplements* as necessary.

Interim Revision Announcements pertaining to this volume of *USP* and *NF* are published in the journal, *Pharmacopeial Forum.* Reprints of *Interim Revision Announcements* are available on order. A single copy of each *Interim Revision Announcement* is available without charge for each subscription to *USP-NF.* Each request should be sent to USPC at the address shown below. Corrections and revisions included in *Interim Revision Announcements* are incorporated in the next regular *Supplement.*

Supplements **to USP 23–NF 18** are issued serially as necessary, with the *Index* in the latest *Supplement* being fully cumulative with respect to all *Supplements* issued previously. Thus, in order to keep the compendia up to date, the user needs to keep all of the *Supplements.* Cumulation of the *Supplements* into a new main volume will occur whenever the amount of text in the *Supplements* becomes unwieldy for the user, or the logistics of publication so dictate. The publication cycle of main volumes may therefore vary from the five-year interval characteristic of new main volumes published since 1950.

Electronic publication of the USP-NF first occurred in November 1992. The complete text of USP is available in a fully searchable electronic format for ease of use. It allows the user to conduct searches that would not have been cost effective or possible with the printed format, and provides fully integrated up-to-date text as it is updated with each *Supplement.*

Inquiries, Comments, and Suggestions

for revisions in the *USP* or *NF* text should be addressed to the Drug Standards Division:

USPC, Inc.
12601 Twinbrook Parkway
Rockville, MD   20852   USA

Telephone: 1-301-881-0666
FAX: 1-301-816-8373
Telex: 710828-9787
Ordering Dept.:   1-800-227-8772

USP-NF Continuous Revision Process

Requests/comments submitted
▼
DSD Subcommittee reviews requests/comments
▼
Publish in *Pharmacopeial Forum* for public review
(*In-process Revision* or *Pharmacopeial Previews*)
▼
Subcommittee approves
▼
DSD Executive Committee approves
▼
Executive Committee of Revision approves
▼
Board of Trustees approves publication in *USP-NF*

1995

Monographs
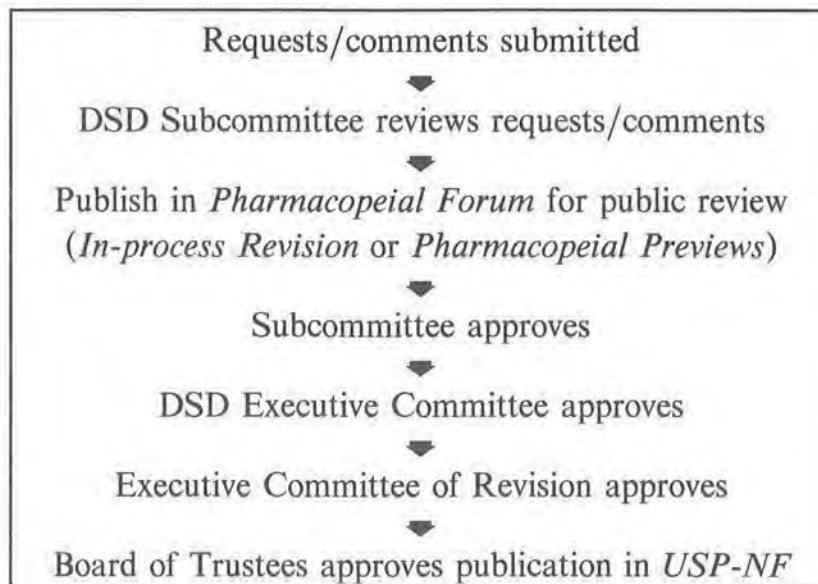
USP 23

NF 18

THE UNITED STATES PHARMACOPEIA

THE NATIONAL FORMULARY

By authority of the United States Pharmacopeial Convention, Inc., meeting at Washington, D.C., March 8–10, 1990. Prepared by the Committee of Revision and published by the Board of Trustees

Official from January 1, 1995

USP
SINCE 1820

UNITED STATES PHARMACOPEIAL CONVENTION, INC.
12601 Twinbrook Parkway, Rockville, MD   20852

NOTICE AND WARNING

Contents

USP 23

NUTRITIONAL SUPPLEMENTS

NF 18

USP 23

THE UNITED STATES PHARMACOPEIA

Official from January 1, 1995

TWENTY-THIRD
REVISION

General Notices and Requirements

Applying to Standards, Tests, Assays, and Other Specifications of the United States Pharmacopeia

Guide to GENERAL NOTICES AND REQUIREMENTS

1

The *General Notices and Requirements* (hereinafter referred to as the *General Notices*) provide in summary form the basic guidelines for the interpretation and application of the standards, tests, assays, and other specifications of the *United States Pharmacopeia* and obviate the need to repeat throughout the book those requirements that are pertinent in numerous instances.

Where exceptions to the *General Notices* are made, the wording in the individual monograph or general test chapter takes precedence and specifically indicates the directions or the intent. To emphasize that such exceptions do exist, the *General Notices* employ where indicated a qualifying expression such as "unless otherwise specified." Thus, it is understood that the specific wording of standards, tests, assays, and other specifications is binding wherever deviations from the *General Notices* exist. By the same token, where no language is given specifically to the contrary, the *General Notices* apply.

TITLE

The full title of this book, including its supplements, is The Pharmacopeia of the United States of America, Twenty-third Revision. This title may be abbreviated to *United States Pharmacopeia*, Twenty-third Revision, or to *USP 23*. The *United States Pharmacopeia*, Twenty-third Revision, supersedes all earlier revisions. Where the term USP is used, without further qualification, during the period in which this Pharmacopeia is official, it refers only to *USP 23* and any supplement(s) thereto.

"OFFICIAL" AND "OFFICIAL ARTICLES"

The word "official," as used in this Pharmacopeia or with reference hereto, is synonymous with "Pharmacopeial," with "USP," and with "compendial."

The designation USP in conjunction with the official title on the label of an article means that the article purports to comply with USP standards; such specific designation on the label does not constitute a representation, endorsement, or incorporation by the manufacturer's labeling of the informational material contained in the USP monograph, nor does it constitute assurance by USP that the article is known to comply with USP standards. The standards apply equally to articles bearing the official titles or names derived by transposition of the definitive words of official titles or transposition in the order of the names of two or more active ingredients in official titles, whether or not the added designation "USP" is used. Names considered to be synonyms of the official titles may not be used for official titles.

Where an article differs from the standards of strength, quality, and purity, as determined by the application of the assays and tests set forth for it in the Pharmacopeia, its difference shall be plainly stated on its label. Where an article fails to comply in identity with the identity prescribed in the USP, or contains an added substance that interferes with the prescribed assays and tests, such article shall be designated by a name that is clearly distinguishing and differentiating from any name recognized in the Pharmacopeia.

Articles listed herein are official and the standards set forth in the monographs apply to them only when the articles are intended or labeled for use as drugs, as nutritional supplements, or as medical devices and when bought, sold, or dispensed for these purposes or when labeled as conforming to this Pharmacopeia.

An article is deemed to be recognized in this Pharmacopeia when a monograph for the article is published in it, including its supplements, addenda, or other interim revisions, and an official date is generally or specifically assigned to it.

The following terminology is used for distinguishing the articles for which monographs are provided: an *official substance* is an active drug entity, a recognized nutrient, or a pharmaceutic ingredient (see also *NF 18*) or a component of a finished device for which the monograph title includes no indication of the nature of the finished form; an *official preparation* is a *drug product*, a *nutritional supplement*, or a *finished device*. It is the finished or partially finished (e.g., as in the case of a sterile solid to be constituted into a solution for administration) preparation or product of one or more official substances formulated for use on or for the patient or consumer; an *article* is an item for which a monograph is provided, whether an official substance or an official preparation.

Nutritional Supplements—The designation of an official preparation containing recognized nutrients as "USP" or the use of the designation "USP" in conjunction with the title of such nutritional supplement preparation may be made only if the article contains two or more of the recognized nutrients and the preparation meets the applicable requirements contained in the individual Class Monograph and General Chapters. Any additional ingredient in such article that is not recognized in the pharmacopeia and for which nutritional value is claimed, shall not be represented nor imply that it is of USP quality or recognized by USP. If a preparation does not comply with applicable requirements but contains nutrients that are recognized in the USP, the article may not designate the individual nutrients as complying with USP standards or being of USP quality without designating on the label that the article itself does not comply with USP standards.

ATOMIC WEIGHTS AND CHEMICAL FORMULAS

The atomic weights used in computing molecular weights and the factors in the assays and elsewhere are those recommended in 1991 by the IUPAC Commission on Atomic Weights and Isotopic Abundances. Chemical formulas, other than those in the Definitions, tests, and assays, are given for purposes of information and calculation. The format within a given monograph is such that after the official title, the primarily informational portions of the text ap-

pear first, followed by the text comprising requirements, the latter section of the monograph being introduced by a boldface double-arrow symbol **»**. (Graphic formulas and chemical nomenclature provided as information in the individual monographs are discussed in the *Preface*.)

ABBREVIATIONS

The term RS refers to a USP Reference Standard as stated under *Reference Standards* in these *General Notices* (see also *USP Reference Standards* ⟨11⟩).

The terms CS and TS refer to Colorimetric Solution and Test Solution, respectively (see under *Reagents, Indicators, and Solutions*). The term VS refers to Volumetric Solution as stated under *Solutions* in the *General Notices*.

The term *PF* refers to *Pharmacopeial Forum*, the journal of standards development and official compendia revision (see *Pharmacopeial Forum* in these *General Notices*).

Abbreviations for the names of many institutions, organizations, and publications are used for convenience throughout *USP* and *NF*. An alphabetized tabulation follows.

Abbreviation	Institution, Organization, or Publication
AAMI	Association for the Advancement of Medical Instrumentation
ACS	American Chemical Society
ANSI	American National Standards Institute
AOAC	AOAC International (formerly Association of Official Analytical Chemists)
ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
CAS	Chemical Abstracts Service
CFR	U.S. Code of Federal Regulations
EPA	U.S. Environmental Protection Agency
FCC	Food Chemicals Codex
FDA	U.S. Food and Drug Administration
HIMA	Health Industry Manufacturers Association
ISO	International Standards Organization
IUPAC	International Union of Pure and Applied Chemistry
NBS	National Bureau of Standards
NIST	National Institute of Standards and Technology (formerly NBS)
USAN	United States Adopted Names
WHO	World Health Organization

Abbreviated Statements in Monographs—Incomplete sentences are employed in various portions of the monographs for directness and brevity. Where the limit tests are so abbreviated, it is to be understood that the chapter numbers (shown in angle brackets) designate the respective procedures to be followed, and that the values specified after the colon are the required limits.

SIGNIFICANT FIGURES AND TOLERANCES

Where limits are expressed numerically herein, the upper and lower limits of a range include the two values themselves and all intermediate values, but no values outside the limits. The limits expressed in monograph definitions and tests, regardless of whether the values are expressed as percentages or as absolute numbers, are considered significant to the last digit shown.

Equivalence Statements in Titrimetric Procedures—The directions for titrimetric procedures conclude with a statement of the weight of the analyte that is equivalent to each mL of the standardized titrant. In such an equivalence statement, it is to be understood that the number of significant figures in the concentration of the titrant corresponds to the number of significant figures in the weight of the analyte. Blank corrections are to be made for all titrimetric assays where appropriate (see *Titrimetry* ⟨541⟩).

Tolerances—The limits specified in the monographs for Pharmacopeial articles are established with a view to the use of these articles as drugs, except where it is indicated otherwise. The use of the molecular formula for the active ingredient(s) named in defining the required strength of a Pharmacopeial article is intended to designate the chemical entity or entities, as given in the complete chemical name of the article, having absolute (100 percent) purity.

A dosage form shall be formulated with the intent to provide 100 percent of the quantity of each ingredient declared on the label. Where the content of an ingredient is known to decrease with time, an amount in excess of that declared on the label may be introduced into the dosage form at the time of manufacture to assure compliance with the content requirements of the monograph throughout the expiration period. The tolerances and limits stated in the definitions in the monographs for Pharmacopeial articles allow for such overages and for analytical error, for unavoidable variations in manufacturing and compounding, and for deterioration to an extent considered acceptable under practical conditions.

The specified tolerances are based upon such attributes of quality as might be expected to characterize an article produced from suitable raw materials under recognized principles of good manufacturing practice.

The existence of compendial limits or tolerances does not constitute a basis for a claim that an official substance that more nearly approaches 100 percent purity "exceeds" the Pharmacopeial quality. Similarly, the fact that an article has been prepared to closer tolerances than those specified in the monograph does not constitute a basis for a claim that the article "exceeds" the Pharmacopeial requirements.

Interpretation of Requirements—Analytical results observed in the laboratory (or calculated from experimental measurements) are compared with stated limits to determine whether there is conformance with compendial assay or test requirements. The observed or calculated values usually will contain more significant figures than there are in the stated limit, and an observed or calculated result is to be rounded off to the number of places that is in agreement with the limit expression by the following pro-

cedure. [NOTE—Limits, which are fixed numbers, are not rounded off.]

When rounding off is required, consider only one digit in the decimal place to the right of the last place in the limit expression. If this digit is smaller than 5, it is eliminated and the preceding digit is unchanged. If this digit is greater than 5, it is eliminated and the preceding digit is increased by one. If this digit equals 5, the 5 is eliminated and the preceding digit is increased by one.

Illustration of Rounding Numerical Values for Comparison with Requirements			
Compendial Requirement	Unrounded Value	Rounded Result	Conforms
Assay limit ≥98.0%	97.96%	98.0%	Yes
	97.92%	97.9%	No
	97.95%	98.0%	Yes
Assay limit ≤101.5%	101.55%	101.6%	No
	101.46%	101.5%	Yes
	101.45%	101.5%	Yes
Limit test ≤0.02%	0.025%	0.03%	No
	0.015%	0.02%	Yes
	0.027%	0.03%	No
Limit test ≤3 ppm	0.00035%	0.0004%	No
	0.00025%	0.0003%	Yes
	0.00028%	0.0003%	Yes

GENERAL CHAPTERS

Each general chapter is assigned a number that appears in brackets adjacent to the chapter name (e.g., ⟨601⟩ *Aerosols*). General chapters that include general *requirements* for tests and assays are numbered from ⟨1⟩ to ⟨999⟩, chapters that are *informational* are numbered from ⟨1000⟩ to ⟨1999⟩, and chapters pertaining to *nutritional supplements* are numbered above ⟨2000⟩.

The use of the general chapter numbers is encouraged for the identification and rapid access to general tests and information. It is especially helpful where monograph section headings and chapter names are not the same (e.g., *Ultraviolet absorption* ⟨197U⟩ in a monograph refers to method ⟨197U⟩ under general tests chapter ⟨197⟩ *Spectrophotometric Identification Tests; Specific rotation* ⟨781S⟩ in a monograph refers to method ⟨781S⟩ under general tests chapter ⟨781⟩ *Optical Rotation;* and *Calcium* ⟨191⟩ in a monograph refers to the tests for *Calcium* under general tests chapter ⟨191⟩ *Identification Tests—General*).

PHARMACOPEIAL FORUM

Pharmacopeial Forum (*PF*) is the USP journal of standards development and official compendia revision. *Pharmacopeial Forum* is the working document of the USP Committee of Revision. It is intended to provide public portions of communications within the General Committee of Revision and public notice of proposed new and revised standards of the *USP* and *NF* and to afford opportunity for comment thereon. The organization of *PF* includes, but is not limited to, the following sections. Subsections occur where needed for Drugs and Pharmaceutic Ingredients and for Nutritional Supplements.

Pharmacopeial Previews—Possible revisions that are considered to be in a preliminary stage of development.

In-process Revision—New or revised monographs or chapters that are proposed for adoption as official USP or NF standards.

Stimuli to the Revision Process—Reports, statements, articles, or commentaries relating to compendial issues.

Nomenclature—Articles and announcements relevant to compendial nomenclature issues and listings of proposed and new United States Adopted Names (USAN) and International Nonproprietary Names (INN).

Interim Revision Announcement (if present)—Official revisions and their effective dates, announcement of the availability of new USP Reference Standards, and announcement of assays or tests that are held in abeyance pending availability of required USP Reference Standards.

Official Reference Standards—Catalog of current lots of USP Reference Standards with ordering information and names and addresses of worldwide suppliers.

REAGENT STANDARDS

The proper conduct of the Pharmacopeial tests and assays and the reliability of the results depend, in part, upon the quality of the reagents used in the performance of the procedures. Unless otherwise specified, reagents are to be used that conform to the specifications set forth in the current edition of *Reagent Chemicals* published by the American Chemical Society. Where such ACS reagent specifications are not available or where for various reasons the required purity differs, compendial specifications for reagents of acceptable quality are provided. (See *Reagents, Indicators, and Solutions*.) Listing of these reagents, including the indicators and solutions employed as reagents, in no way implies that they have therapeutic utility; furthermore, any reference to USP or NF in their labeling shall include also the term "reagent" or "reagent grade."

USP REFERENCE STANDARDS

USP Reference Standards are authentic specimens that have been approved by the USP Reference Standards Committee as suitable for use as comparison standards in USP or NF tests and assays. (See *USP Reference Standards* ⟨11⟩.) Currently official lots of USP Reference Standards are published in *Pharmacopeial Forum*.

JOINT APPENDIX 40

United States Patent [19]

Martin et al.

[11] **Patent Number:** 4,737,323

[45] **Date of Patent:** Apr. 12, 1988

[54] **LIPOSOME EXTRUSION METHOD**

[75] Inventors: **Francis J. Martin,** San Francisco; **Jacqueline K. Morano,** Mountain View, both of Calif.

[73] Assignee: **Liposome Technology, Inc.,** Menlo Park, Calif.

[21] Appl. No.: **829,710**

[22] Filed: **Feb. 13, 1986**

[51] Int. Cl.⁴ **A61K 9/52; B01J 13/02**
[52] U.S. Cl. **264/4.3;** 210/500.23; 210/500.26; 424/420; 424/450; 428/402.2; 436/829; 514/34
[58] **Field of Search** 264/4.3; 428/402.2; 424/19, 38, 420, 450; 436/829; 210/500.23, 500.26

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Chemistry and Physics of Lipids 12, (1973), 75–95 pp., North–Holland Publ. Co., "Studies on Phosphatidylcholine Model Membranes I., Size–Heterogeneity Effect on Permeability Mesurement, by S. E. Schullery & J. P. Garzaniti, Chem. Dept. Eastern Mich. U., U.S.A.

Primary Examiner—Richard D. Lovering
Attorney, Agent, or Firm—Ciotti & Murashige, Irell & Manella

[57] **ABSTRACT**

A suspension of liposomes whose sizes are predominantly greater than about 1 micron is passed through an asymmetric ceramic filter whose inner-surface pore size is about 1 micron. The processed liposomes have a selected average size of about 0.4 microns or less, depending on the number of filter cycles, and a narrow distribution.

7 Claims, 1 Drawing Sheet

JA001079

FIG. 1

FIG. 2

FIG. 2A

FIG. 3

To drain

To free-drug removal
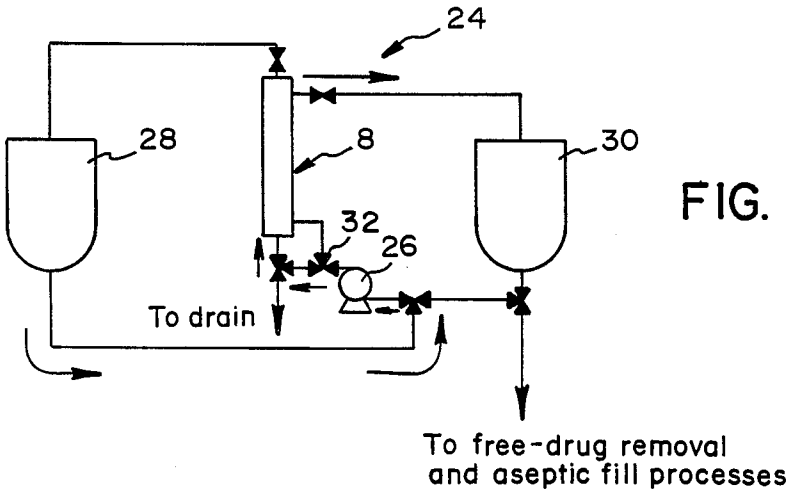and aseptic fill processes

JA001080
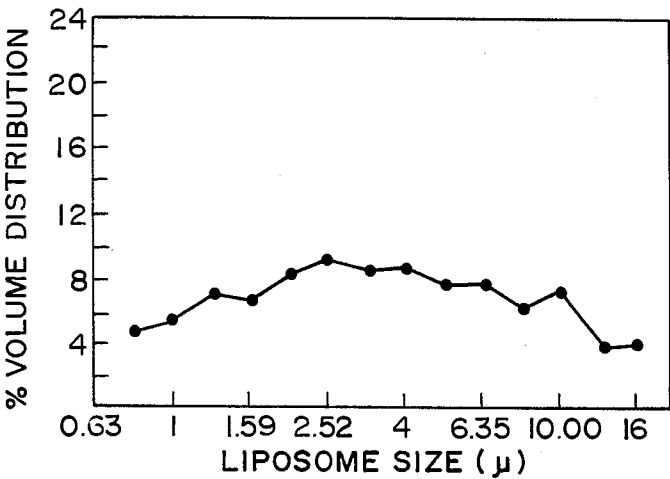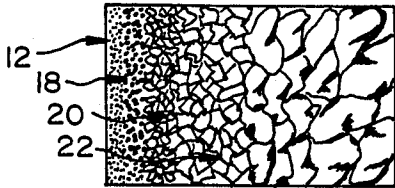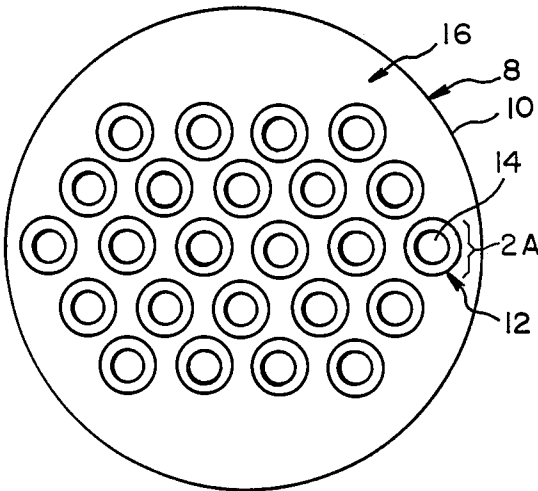
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LIPOSOME EXTRUSION METHOD

FIELD OF THE INVENTION

The present invention relates to methods for producing liposomes in a selected size range, preferably between about 0.1 and 0.4 microns.

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BACKGROUND OF THE INVENTION

The use of liposomes for drug delivery has been proposed for a variety of drugs, particularly those which are administered parenterally. Liposomes have the potential for providing controlled "depot" release of the administered drug over an extended time period, and of reducing side effects of the drug, by limiting the concentration of free drug in the bloodstream. Liposomes can also alter the tissue distribution and uptake of drugs, in a therapeutically favorable way, and can increase the convenience of therapy, by allowing less frequent drug administration. Liposome drug delivery systems are reviewed in Poznansky.

Generally, the optimal liposome size for use in parenteral administration is between about 0.1 and 0.3, and up to 0.4, microns. Liposomes in this size range can be sterilized by passage through conventional filters having particle size discrimination of about 0.2 microns. This size range of liposomes also favors biodistribution in certain target organs, such as liver, spleen, and bone marrow (Gabizon), and gives more uniform and predictable drug-release rates and stability in the bloodstream. Liposomes whose sizes are less than about 0.4 microns also show less tendency to agglutinate on storage, and are thus generally safer and less toxic in parenteral use than larger-size liposomes.

A variety of techniques have been proposed for preparing liposomes, including drug-containing liposomes (Szoka 1983). Typically, these methods yield liposomes which are heterodisperse, and predominantly greater than about 1 micron in size. These initial heterodisperse suspensions can be reduced in size and size distribution by a number of known methods. One size-processing method which is suitable for large-scale production is homogenization. Here the initial heterodisperse liposome preparation is pumped under high pressure through a small orifice or reaction chamber. The suspension is usually cycled through the reaction chamber until a desired average size of liposome particles is achieved. A limitation of this method is that the liposome size distribution is typically quite broad and variable, depending on a number of process variables, such as pressure, number of homogenization cycles, and internal temperature. Also, the processed fluid has the potential to pick up metal and oil contaminants from the homogenizer pump, and may be further contaminated by residual chemical agents used to sterilize the pump seals.

Sonication, or ultrasonic irradiation, is another method that is used for reducing liposome sizes. This technique is useful especially for preparing small uni-

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lamellar vesicles (SUVs), in the 0.025–0.08 micron size range. However, a narrow size distribution of liposomes can only be achieved at liposome sizes of about 0.05 microns, i.e., when the liposomes have been substantially completely reduced in size. The very small liposomes have limited drug capacity and less favorable biodistribution properties than those in the 0.1–0.4 micron size range, as noted below. The processing capacity of this method is also quite limited, since long-term sonication of relatively small volumes is required. Also, heat build-up during sonication can lead to peroxidative damage to the lipids, and sonic probes shed titanium particles which are potentially quite toxic in vivo.

A third general size-processing method known in the prior art is based on liposome extrusion through uniform pore-size polycarbonate membranes (Szoka 1978). This procedure has advantages over the above homogenization and sonication methods in that a variety of membrane pore sizes are available for producing liposomes in different selected size ranges, and in addition, the size distribution of the liposomes can be made quite narrow, particularly by cycling the material through the selected-size filter several times. Nonetheless, the membrane extrusion method has several drawbacks in large-scale processing. For one, the pores in the membrane tend to clog, particularly when processing concentrated suspensions and/or when the liposome sizes are substantially greater than the membrane pore sizes. The clogged membranes cannot be cleared, because the filter housing configuration does not allow back flushing, and replacing the filter is likely to compromise the sterility of the extrusion operation. Secondly, the membranes themselves are planar disks which must be mounted against a flat mechanical support. This severely restricts the surface area available for extrusion, and leads to slow throughput. Although the problems of clogging and slow throughput can be overcome partially at high extrusion pressures, such requires specially adapted filter holders and membrane tearing become more of a problem. Finally, polycarbonate membranes cannot be steam-sterilized in place, with a high degree of confidence, due to their inherent fragility.

BACKGROUND OF THE INVENTION

It is therefore a general object of the invention to provide a novel liposome size-processing method which overcomes the above-mentioned limitations and problems associated with the prior art.

One specific object of the invention is to provide such a method which yields sized liposomes having a selected average size of between about 0.1 to 0.4 microns, and a relatively narrow distribution of sizes.

Still another object of the invention is to provide such a method which can be operated in a relatively problem-free manner, without heat build-up, at high throughput volumes, and in a large-scale operation.

Providing such a method which can be practiced with little risk of contamination and under sterile conditions is yet another object of the invention.

In practicing the method of the invention, a suspension of liposomes containing a substantial portion of liposomes with sizes greater than about 1 micron, are passed through an asymmetric ceramic filter having an inner-side pore size of about 1 micron. The resulting liposomes have an average particle size of between about 0.2 and 0.4 microns, depending on the number of

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times the liposomes are cycled through the membrane, and a standard size distribution of about 30–45%.

The suspension may be alternately passed through the membrane, in an outside-to-inside directions, to maintain the membrane in an unclogged condition, allowing high throughput processing, even for a concentrated suspension of liposomes.

The liposome average size may be further reduced by passage through similar types of ceramic filters, but which have smaller specified inner-surface pore sizes.

These and other objects and features of the present invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph showing the size distribution of multiple lamellar vesicles (MLVs) prior to size-processing according to the invention;

FIG. 2 is a sectional view of a filter apparatus of the type used in the present invention, with the inset showing an enlarged inner wall portion of a filter in the apparatus; and

FIG. 3 is a flow diagram of a liposome processing system for preparing liposomes according to the present invention.

DETAILED DESCRIPTION OF THE INVENTION

I. Preparation of Liposome Suspension

A. Unsized Liposomes

The liposomes, or lipid vesicles, of the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. A variety of lipids having selected acyl chain compositions are commercially available or may be obtained using standard lipid isolation procedures. The selection of lipids for therapeutic liposomes containing an active drug is generally guided by considerations of (a) drug-entrapment efficiency, (b) drug-release rate in serum, and (c) biodistribution and targeting properties. These considerations are discussed at length, for example, in U.S. patent application Ser. No. 806,084 for "Liposome/Anthraquinone Drug composition and Method", filed Dec. 6, 1985.

Several methods for producing a suspension of the heterogeneous-size vesicles are available. In one preferred method, vesicle-forming lipids are taken up in a suitable organic solvent or solvent system, and dried in vacuo or under an inert gas to a lipid film. Where the vesicles are formulated to include a lipophilic or amphiphilic drug, such may be included in the lipids forming the film. To form the vesicles, aqueous medium is added to the dry film, and the film is allowed to hydrate, typically over a one-two hour period with gentle shaking. The lipids hydrate to form multilamellar vesicles (MLVs) whose sizes range typically between about 0.5 microns to about 10 microns or greater. In general, the size distribution of MLVs can be shifted toward slightly smaller sizes by hydrating the lipids under more vigorous shaking conditions. The aqueous medium used in hydrating the lipid film may include a water-soluble drug which then becomes encapsulated in the vesicles which form during lipid hydration.

Example I below described describes the preparation of an MLV suspension whose size distribution is shown in FIG. 1. The lower size range of about 0.8 was the

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lower limit of size discrimination of the particle-sizer used. As seen, a suspension contained a broad range of sizes up to about 16 microns, with average sizes between about 2–4 microns.

For producing liposomes under conditions of high encapsulation efficiency, the reverse evaporation phase method first described by Szoka 1978 is preferred. The reverse-phase evaporation vesicles (REVs) formed by this method are characterized by (a) one or a few bilayers, (b) an encapsulation efficiency typically between about 20–50%, and (c) a broad spectrum of sizes between about 0.5 and up to 20 microns. These and other liposome-preparation methods have been reviewed extensively (Szoka 1980).

B. Sizing Liposomes

According to an important feature of the invention, the unsized liposomes are passed through an asymmetric ceramic filter, to produce liposomes with a selected average size between about 0.1 and 0.4 microns, and a narrow distribution of liposome sizes. A preferred ceramic filter is a Ceraflow TM Microfilter available commercially from the Norton Company (Worcester, MA), and supplied as a multifilter cartridge-type filter apparatus, such as seen cross-sectionally in FIG. 2. The filter apparatus 8 includes a tubular casing 10 which houses a plurality of tubular filters, such as filter 12, a side-wall portion of which is shown in enlarged view in the inset in the figure. The casing is provided with an inlet manifold (not shown) through which the liposome suspension can be supplied under pressure to the inner tubular region of each filter, such as inner region 14 of filter 12. The material, on passage through the filters, is collected from an extratubular space 16, through a casing outlet (also not shown). A useful operational feature of the filter system just described is the ability to filter in either direction, that is, in a forward, inside-to-outside direction or in a back, outside-to-inside direction in which material is pumped under pressure from the extratubular space into the filter interior regions, and collected at the casing manifold. Back direction flow may alternate with forward direction to reduce the tendency of the filters to clog.

The asymmetric construction of the filters is seen in the inset in FIG. 2, which shows an enlarged sectional view taken through a wall portion of filter 12. The filter is composed of a series of controlled-thickness ceramic layers or strata, such as layers 18, 20, and 22, arranged coaxially about the filter's inner tubular space. The layers are each composed of sintered particles, with the inner wall having the smallest particles and the outer walls having progressively larger particles. The particles forming the inner walls are dimensioned to provide a defined surface pore size in the sintered inner layer. For example, the Ceraflow TM filters supplied by Norton have surface pore sizes of either 1.0, 0.45, or 0.2 microns, and are designed for filtering particles, in a fluid flowing through the filters in an inside-to-outside direction, whose size is equal to or greater than the rated pore size.

FIG. 3 shows an extrusion system 24 employing a cartridge-type filter apparatus 8 of the type just described. The system includes a pump 26, and a pair of vessels 28, 30, which hold the liposome suspension being processed. The pump is connected to the vessels through a valving arrangement which includes a series of valves such as valve 32, for effecting fluid flow either in a forward direction (the direction of arrows in FIG.

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3) from vessel **28**, through the apparatus in an inside-to-outside direction, to vessel **30**, or in the reverse, back direction. In an alternative system, the driving pressure is created by compressed gas which is connected to the vessels in a conventional manner. From vessel **30**, the processed liposome suspension can be transferred to a sterile fill system, as indicated.

In a typical processing operation, a suspension of heterogeneous size liposomes are placed in vessel **28**, and the valves are set initially to pump the suspension through the filter apparatus in a forward direction. As will be seen from the procedure described in Examples II and III, and according to an important finding of the present invention, a single passage through the 1.0 micron pore size filter apparatus reduces the average liposome size to about 0.3–0.35 microns, with a standard size deviation of about 40%. These size characteristics are suitable for purposes of subsequent filter sterilization and to desirable therapeutic properties. Alternatively, the suspension may be recycled through the filter apparatus, and preferably by alternating the flow in forward and backward directions, to reduce the average size of the liposomes selectively. For example, as described in Example II, cycling the above MLV suspension through the 1.0 pore-size filter several times gradually reduced the liposome average size from 0.3 microns (after one filtration) to about 0.2 microns (after several passes). Cycling the material alternately in a back direction acts to prevent particle build-up and clogging at the filter's inner surface.

In the filter operation described in Example III, liposome average sizes were reduced to about 0.35 microns after a single pass through a 1 micron filter and further reduced to about 0.27 microns with three passes.

If smaller liposome sizes are desired, the material can be further processed by passage through similar types of asymmetric filters having inner surface pore sizes of 0.45 or 0.2 microns. Example III shows the gradual reduction in pore size in liposomes after initial sizing using a 1 micron filter, by five passes through a 0.45 micron filter. As seen, repeated extrusion through the 0.45 micron filter reduced average liposome sizes to about 0.2 microns.

Alternatively, the material may be processed by direct passage through a smaller pore size ceramic filter (less than about 0.5 micron), to achieve direct reduction of heterogeneous-size liposomes to average sizes of about 0.2 microns or less. However, since filter clogging tends to occur when unsized liposomes are pumped through ceramic filters with smaller pore sizes, it may be necessary to increase filtration pressure, use a more dilute liposome suspension, flow the material through the filter initially in a back (outside-to-inside) direction, and/or alternate the direction of flow more frequently to achieve high-volume throughput. It is noted that smaller-pore filters are generally not needed, since direct reduction in liposome size to a size range that is suitable for parenteral use (0.2–0.3 microns) can be achieved directly, and at high throughput rates, with a 1 micron pore-size filter.

C. Filter Sterilization and Free-Drug Removal

The size-processed liposome suspension may be readily sterilized by passage through a sterilizing membrane having a particle discrimination size of about 0.2 microns, such as a conventional 0.22 micron depth membrane filter. The sterilizing filter may be an asymmetric ceramic filter of the type described above, but having an inner surface pore size of about 0.2. However,

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since an asymmetric filter will produce some liposome sizing effect, with the attendant possibility of higher pressure requirements and/or eventual membrane clogging, a conventional membrane filter is preferred for sterilization. Also, the tortuous path pore structure of conventional sterilizing membrane filters is preferred for maximum bacteria retention.

Where liposomes are formulated to contain an entrapped drug, for use in parenteral drug administration, it is usually advantageous to further process the sized liposomes to remove free drug, i.e., drug present in the bulk aqueous phase of the suspension. This is done to reduce the effects of free drug and to maximize the benefits achievable by drug entrapment in the liposomes. Free drug may be present in a substantial amount in the case of a water-soluble drug, which can be encapsulated at a maximum efficiency of about 50%, as noted above, or in the case of a lipophilic or amphiphilic drug which has originally been included in vesicle-forming lipids in molar excess of the liposome drug-carrying capacity, as a strategy for maximizing the drug/lipid ratio in the liposomes. It may also be desirable to reduce the bulk phase concentration of other solute molecules, such as carbohydrates, chelate agents, or the like, used in preparing the liposomes but not desired in parenteral administration.

Several methods are available for removing free drug from a liposome suspension. The sized liposome suspension can be pelleted by high-speed centrifugation, leaving free drug and very small liposomes in the supernatant. Another method involves concentrating the suspension by ultrafiltration, then resuspending the concentrated liposomes in a drug-free replacement medium. Alternatively, gel filtration can be used to separate larger liposome particles from solute (free drug) molecules. Ion-exchange chromatography may provide an efficient method of free drug removal, in instances where a suitable drug-binding resin can be identified. One preferred method of free drug removal is by diafiltration, using a conventional hollow fiber or stacked filter device, which preferably has a molecular weight cutoff of between about 10,000–100,000 daltons. Diafiltration has the advantage that it can be used in-line in a sterile liposome-processing system of the type shown in FIG. 3.

II Utility

Sized liposome suspensions prepared according to the invention are useful in a variety of liposome therapeutic compositions in which controlled sizes between about 0.1 and 0.3 microns, and within a narrow size distribution, are desired. One important class of compositions is drug-containing liposomes, for parenteral drug administration. As indicated above and reviewed extensively in the Poznansky reference, liposomal drug-delivery systems have been developed and tested with a wide range of water-soluble and lipid-soluble drugs. Although many of the earlier proposed liposome/drug systems were not carefully defined in terms of size, a variety of experimental evidence and practical considerations indicate advantages of the 0.1 to 0.3 micron size range. This size range is generally preferred to larger-size liposomes, as indicated above, because of ease of sterilization, improved biodistribution, more size uniformity, and less tendency to aggregate on storage.

With liposome sizes below about 0.1 microns, the drug-carrying capacity of the liposomes, measured either by internal encapsulation of lipid-bilayer volume,

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become somewhat restrictive. Also, as liposome sizes are reduced below about 0.1 microns, the liposomes appear to behave more like free drug in terms of biodistribution and drug-clearance rates. The effect of liposome size on pharmacokinetic properties of liposomes carrying the anti-tumor drug doxorubicin has been examined in connection with the drug-liposome invention described in the above mentioned U.S. patent application Ser. No. 806,084 for "Liposome/Anthraquinone Composition and Method", filed Dec. 6, 1985. As detailed there, liposomes with average sizes of about 0.035 microns were much more similar to free drug in biodistribution and drug clearance rates than liposomes with average sizes of about 0.115 microns.

The present invention offers a number of advantages over prior art liposome-sizing methods. The ceramic filter can be sterilized by dry heat at temperatures which are effective to destroy endotoxins, and the system is compatible with a variety of solvents, including many organic solvent which are not tolerated by polycarbonate-type membranes. The method generates very little heat, and can be performed under aseptic conditions.

The liposome processing method yields liposome sizes in a selected size range of between about 0.1 and 0.4 microns, and with a relatively narrow distribution of sizes i.e., uniform liposome sizes. The method is well suited to a high throughput liposome processing operation which is reliable and requires very little maintenance, such as filter cartridge replacement. High throughput is due in part to the relatively high pressure which may be used, and in part because bidirectional operation reduces clogging problems. High throughput is also due to the surface area of membrane available. A tubular cartridge configuration is more efficient in terms of membrane surface area and makes the process easily scalable.

The finding that liposomes may be directly and efficiently reduced from heterogeneous sizes predominantly greater than 1 micron, to a narrow distribution of sizes in a selected size range between about 0.2 and 0.3 microns, using a filter with an inner-wall pore size of 1 micron, allows for direct liposome sizing without the need to pass the liposome suspension through a series of progressively smaller pore-size membranes, as has been generally found for polycarbonate membranes.

The following examples illustrate both use and results achievable with the method of the invention, but are in no way intended to limit the scope of the invention.

EXAMPLE I

Preparation of Heterogeneous-Size Liposomes

Phosphatidylcholine (PC) was obtained from Asahi Lipids (Japan), cholesterol (CH) from Sigma Chemical Co. (St. Louis, MO), and phosphatidylglycerol (PG) were obtained from Avanti Lipid (Birmingham, AL). PC (0.12 moles), CH (0.09 moles), and PG (0.01 moles) were dissolved in 260 ml of chloroform, and the solvent was removed by rotary evaporation under reduced pressure, leaving a thin film of lipid in the flask. One liter of hydration buffer consisting of 10.7 mM $NaH_2PO_4.H_2O$, 48.4 mM $Na_2HPO_4.7H_2O$, pH 7.4, and 86.1 mM NaCl was added to the flask and swirled gently over the lipids. The lipids were allowed to swell gently for about 2 hours.

The size distribution of a typical suspension made as described above was measured in a Coulter Counter, Model TA2, using a 50 micron aperture tube. The

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counter device is capable of discriminating size between about 0.8 and 20 microns, and is programmed to express each size window as a percentage of volume distribution, based on a 100% volume distribution in the 0.8–20 micron size range. The size-distribution curves for the liposomes are shown in FIG. 1. It is noted that only those liposomes whose sizes are more than about 0.8 microns are included in the normalized curves. That is, the size distribution curves do not show actual volume percentages below 0.8 micron sizes. As seen, the liposomes have a broad distribution over the size range 0.8 to greater than 16 microns, with an average size of between about 2–4 microns.

EXAMPLE II

Liposome Size Processing

An asymmetric ceramic filter apparatus having a specified inner surface pore size of 1.0 micron was obtained from the Norton Company (Worcester, MA). The filter was connected in a two-vessel system of the type shown in FIG. 3, but using a pressurized nitrogen supply source to pump fluid from one vessel to the other through the filter apparatus.

The liposome suspension from Example I was added to the first vessel, and the vessel was pressurized with filtered nitrogen gas to about 200–250 psi. The valve arrangement connecting the two vessels was first adjusted to pump the suspension through the filter apparatus, in a forward, inside-to-outside direction, into the second vessel. The valving in the system was then reversed to pump the suspension through the filter apparatus in a back direction. The material was filtered an additional eight times, four times in a forward direction and four times alternately in a back direction, with sample material being removed after each step for later size-distribution determination.

The size distribution of the liposomes for each of the ten samples was determined using a conventional particle sizer calibrated with latex particle size standards. From the measured sizes of the sample, the machine calculates mean particle diameter and percent standard deviation with respect to the mean values. The data are shown in Table 1 below. Odd number passes were in a forward direction through the filter apparatus, and even number passes were in a back direction.

TABLE 1

No. of Passes*	Mean Diam. (nm)	Std. Dev. (%)
1	301.4	36.5
2	300.7	38.1
3	259.4	32.6
4	251.8	36.2
5	239.7	34.2
6	241.8	33.4
7	233.1	33.6
8	234.6	31.1
9	233.2	33.4
10	223.5	35.1

The data show a gradual reduction in average liposome size, with increasing filtration steps, from about 0.3 to 0.2 microns. The extent of size reduction produced by each filtration step appears to be greater in the forward direction than in the back direction. Interestingly, the standard deviation of sizes was not improved appreciably by repeated passages through the filter.

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EXAMPLE III

Liposome Size Processing

The liposome suspension from Example I was added to one vessel in a two-vessel system of the type shown in FIG. 3, and the valve arrangement connecting the two vessels was adjusted to pump the suspension through a 1 micron ceramic filter apparatus (Example II), in a forward, inside-to-outside direction, into the second vessel. The valving in the system was then reversed to pump the suspension through the filter apparatus in a back direction. The material was passed through the filter a third time in a forward direction. The 1μ filter was then replace with a 0.45μ ceramic filter (Norton Company) and the material pumped through the apparatus three times in a forward direction (passes 4, 6, and 8) and two times in a back direction (passes 5 and 7). The size distribution of the liposomes after each pass was determined as in Example II. The data are shown in Table 2 below, along with the gauge pressure, in psi, used at each pass.

TABLE 2

No. of Passes	Filter Size	Pressure (psi)	Mean Diam. (nm)	Stan. Dev. (%)
1	1μ	400	344.3	42
2	1μ	75	295.8	35
3	1μ	250	269.2	35
4	0.45μ	150	234.0	33
5	0.45μ	75	242.0	32
6	0.45μ	250	215.1	31
7	0.45μ	70	210.9	31
8	0.45μ	150	210.4	29

The data show a gradual reduction in average liposome size, with increasing filtration steps, from about 0.35 to 0.2 microns. The gradual reduction in size, after replacing the 1 micron filter with a 0.45 micron filter, is not significantly greater than that achieved in Example II using a 1 micron filter only. The data also show that

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the required filtration pressure was substantially greater in the forward than in the back direction.

While preferred embodiments of the invention have been described herein, it will be apparent to those skilled in the art that various changes and modifications can be made without departing from the invention.

It is claimed:

1. A method of producing a suspension of liposomes which have uniform sizes and a selected average size of less than about 0.4 microns, said method comprising:

   providing a suspension of heterogeneous-size liposomes containing a substantial portion with sizes greater than 1.0 micron in size, and

   passing the suspension under pressure through an asymmetric ceramic filter whose inner-surface pore size is greater than the desired average liposome size and no greater than about 1 micron.

2. The method of claim 1, wherein the liposome suspension is passed through the filter in an inside-to-outside direction.

3. The method of claim 1 wherein the membrane is a Ceraflow TM asymmetric ceramic filter.

4. The method of claim 1, for producing a suspension of liposomes having a selected average size of between about 0.3 and 0.4 microns, wherein said asymmetric filter has an inner-surface pore size of about 1 micron.

5. The method of claim 1, for producing a suspension of liposomes having a selected average size of between about 0.2 and 0.3 microns, wherein said asymmetric filter has an inner-surface pore size of about 0.45 microns in size.

6. The method of claim 5, for producing a suspension of liposome having a selected average size between about 0.2 and 0.3 microns, which further includes passing the suspension repeatedly through the filter, in an inside-to-outside direction, until the desired liposome average size is achieved.

7. The method of claim 6 which further includes alternately passing the suspension through the filter in an outside-to-inside direction.

* * * * *

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JOINT APPENDIX 41

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(12) **Patent Application Publication**   (10) Pub. No.: **US 2006/0240554 A1**

Chen et al.   (43) Pub. Date: **Oct. 26, 2006**

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(54) **LIPID NANOPARTICLE BASED COMPOSITIONS AND METHODS FOR THE DELIVERY OF BIOLOGICALLY ACTIVE MOLECULES**

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(21) Appl. No.: **11/353,630**

(22) Filed: **Feb. 14, 2006**

Related U.S. Application Data

(60) Provisional application No. 60/652,787, filed on Feb. 14, 2005. Provisional application No. 60/678,531, filed on May 6, 2005. Provisional application No. 60/703,946, filed on Jul. 29, 2005. Provisional application No. 60/737,024, filed on Nov. 15, 2005.

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C07C   *229/10*   (2006.01)

(52) U.S. Cl. 435/375; 554/110

(57) **ABSTRACT**

The present invention relates to novel cationic lipids, transfection agents, microparticles, nanoparticles, and short interfering nucleic acid (siNA) molecules. The invention also features compositions, and methods of use for the study, diagnosis, and treatment of traits, diseases and conditions that respond to the modulation of gene expression and/or activity in a subject or organism. Specifically, the invention relates to novel cationic lipids, microparticles, nanoparticles and transfection agents that effectively transfect or deliver biologically active molecules, such as antibodies (e.g., monoclonal, chimeric, humanized etc.), cholesterol, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, dsRNA, allozymes, aptamers, decoys and analogs thereof, and small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules, to relevant cells and/or tissues, such as in a subject or organism. Such novel cationic lipids, microparticles, nanoparticles and transfection agents are useful, for example, in providing compositions to prevent, inhibit, or treat diseases, conditions, or traits in a cell, subject or organism. The compositions described herein are generally referred to as formulated molecular compositions (FMC) or lipid nanoparticles (LNP).
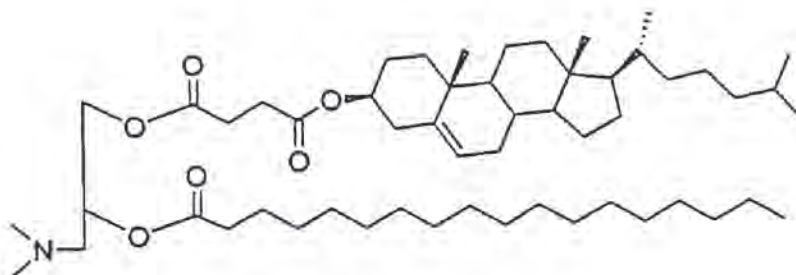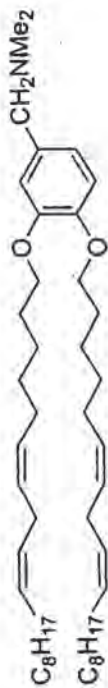
Figure 1
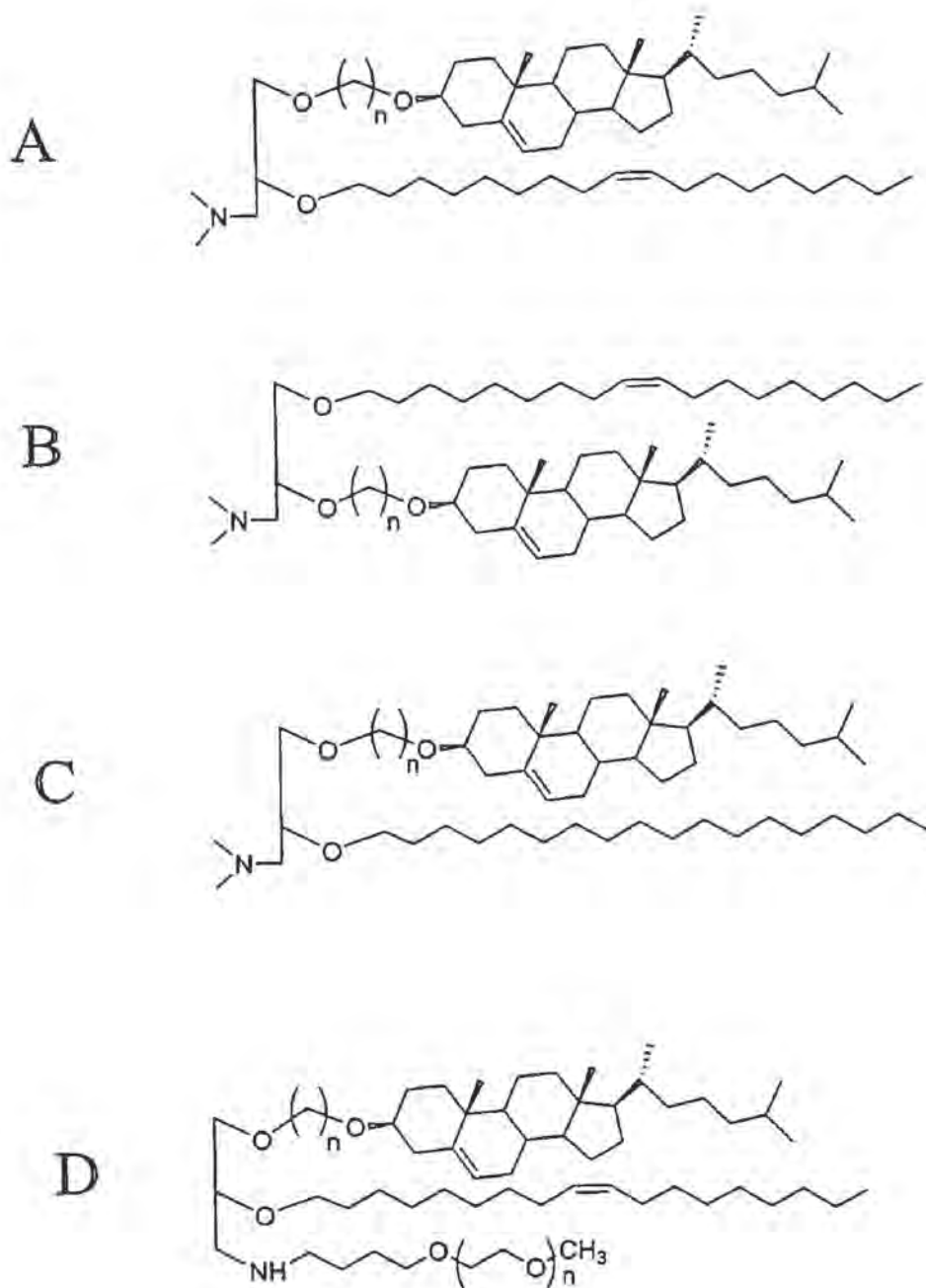
Figure 2
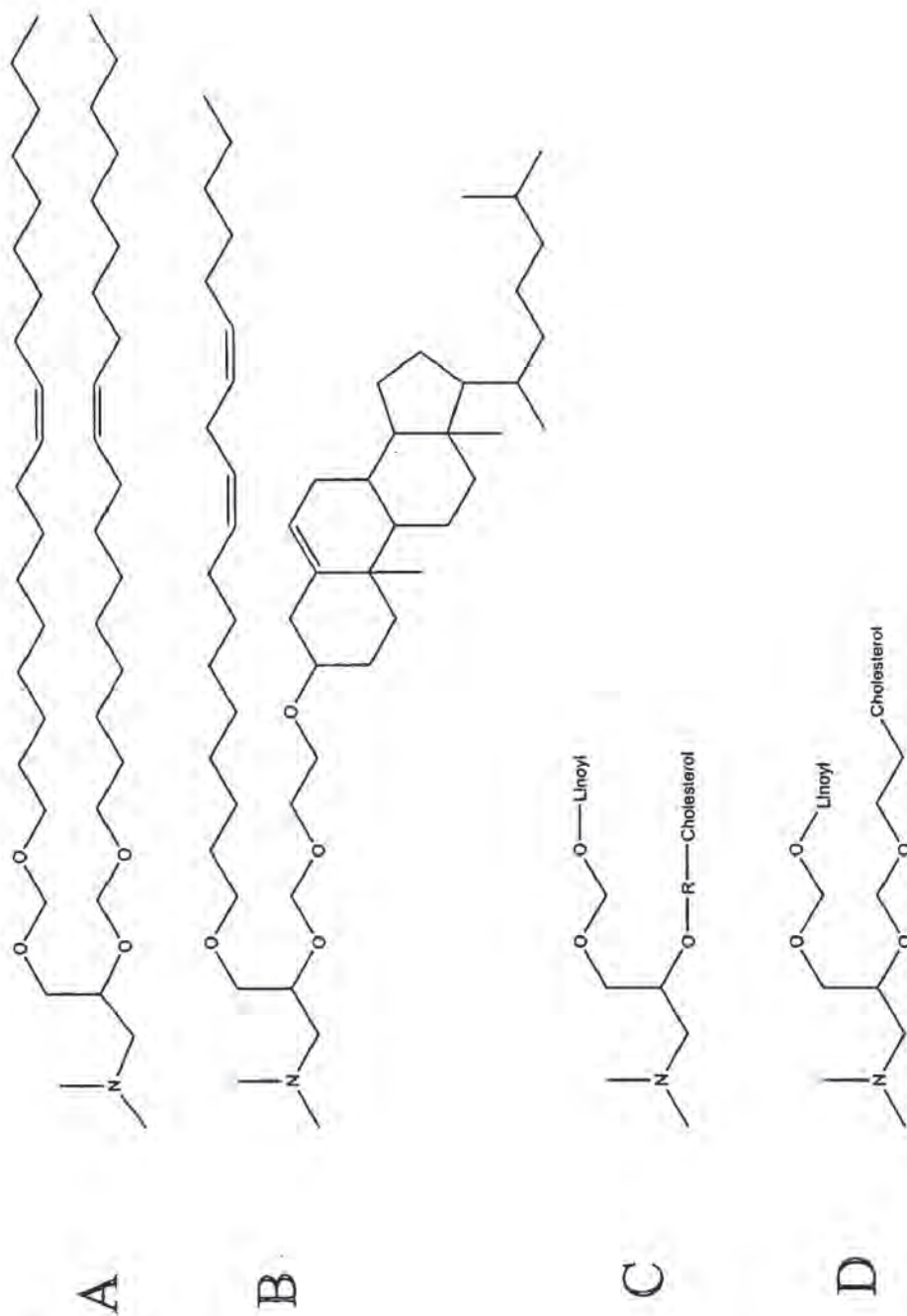
Figure 3

A

B

C

D
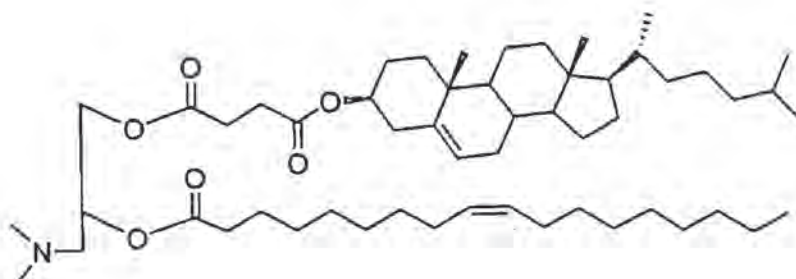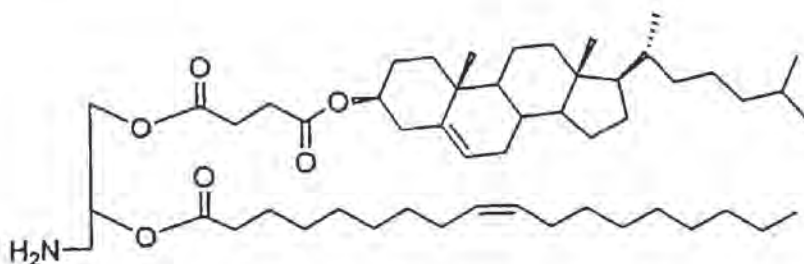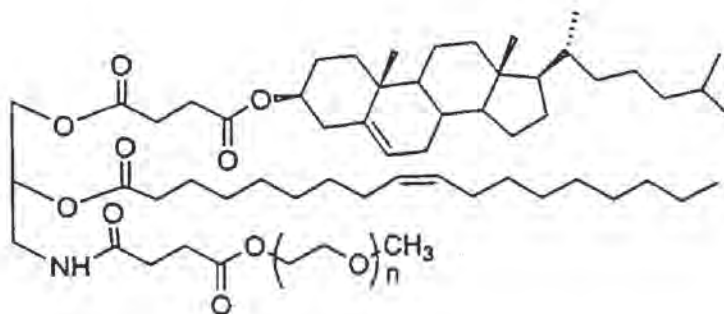
Figure 4
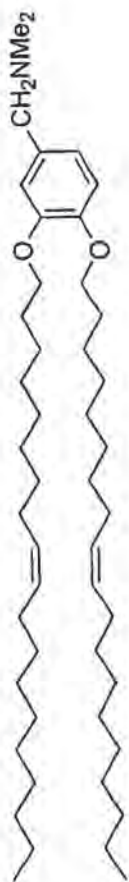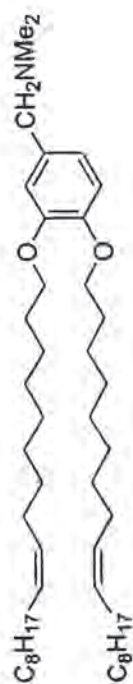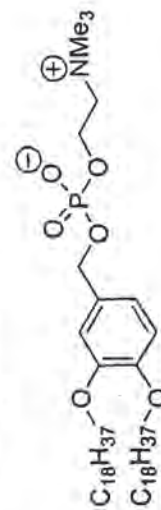
FIGURE 5

Cationic Lipids

FIGURE 6

FIGURE 7

Inverted Hexagonal Structure

Lipid

Nucleic acid

Lamella Structure

Lipid bilayer

Nucleic acid
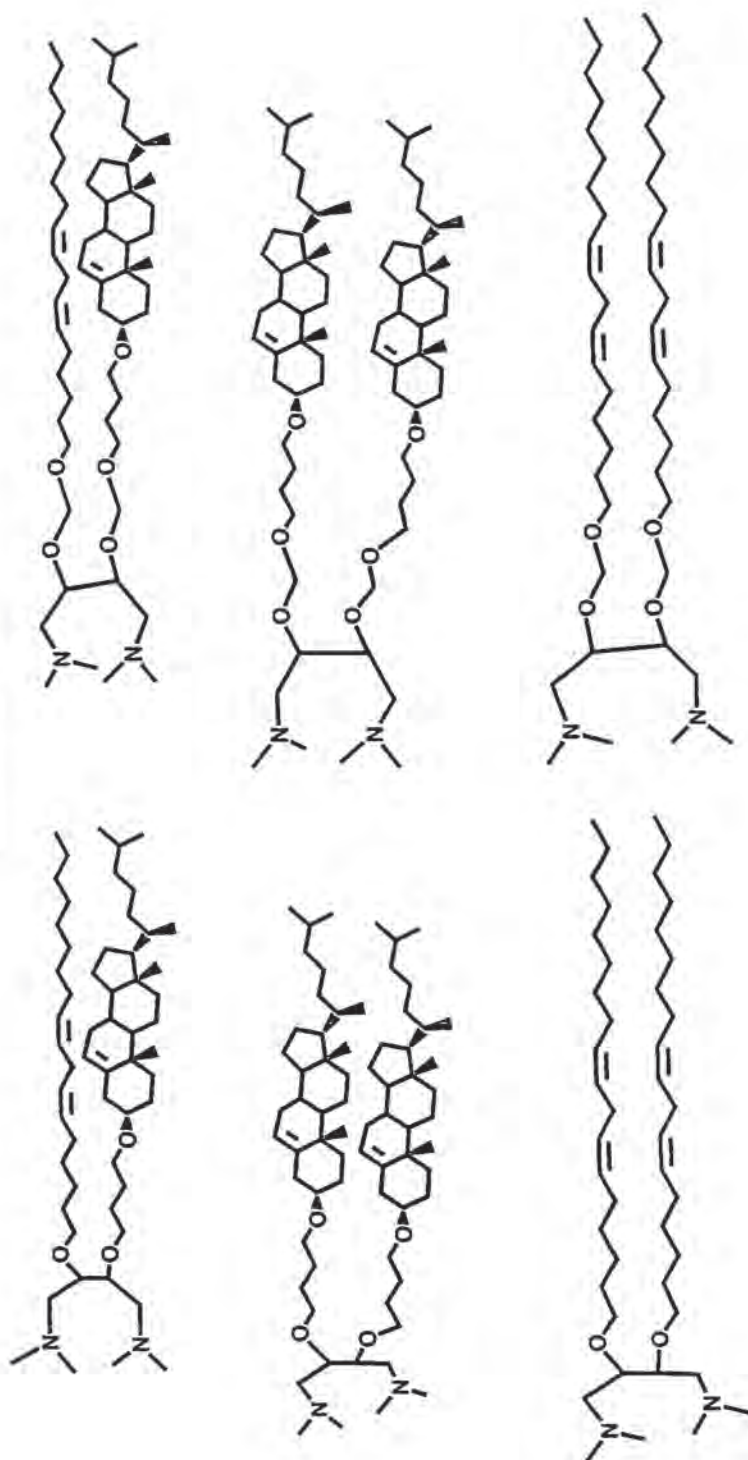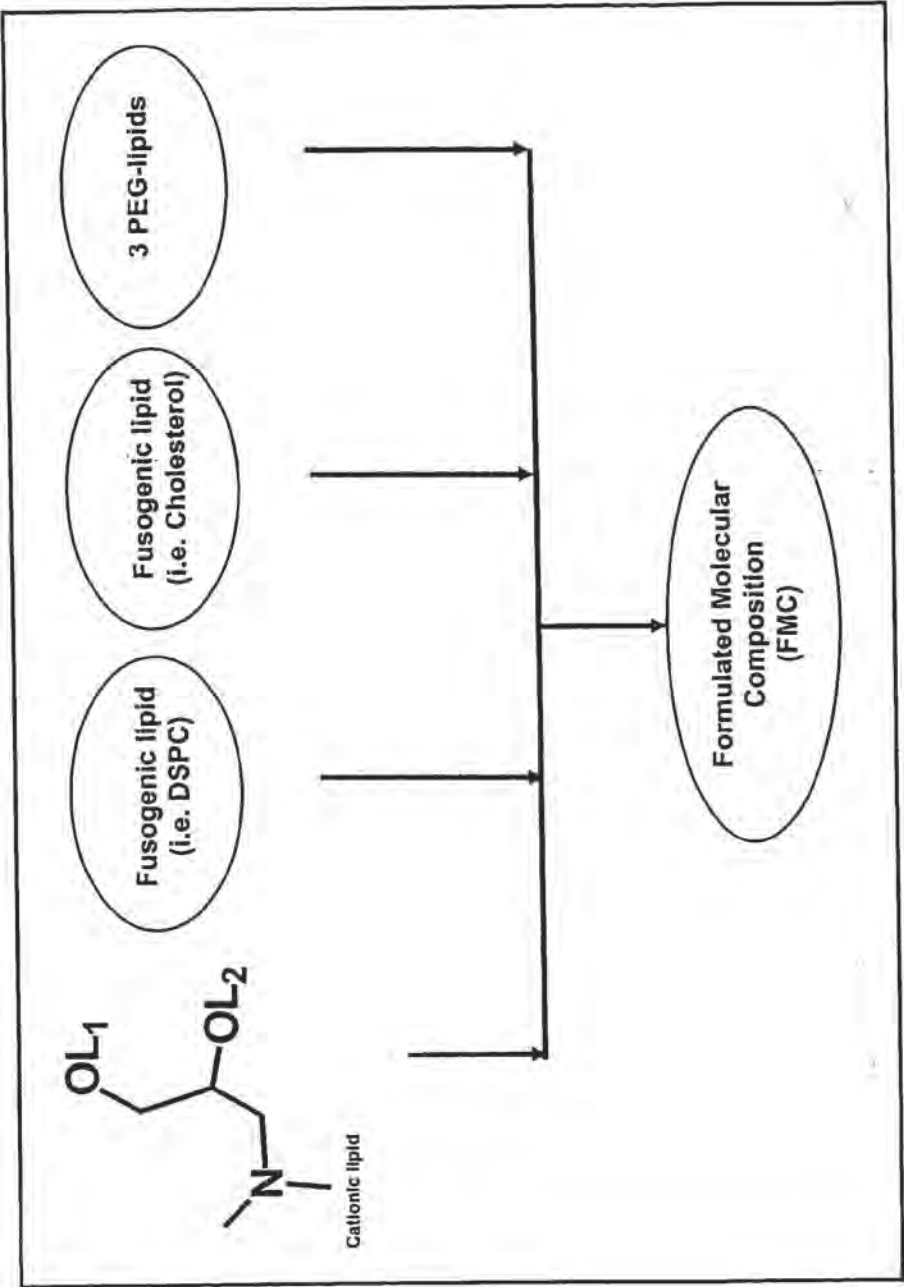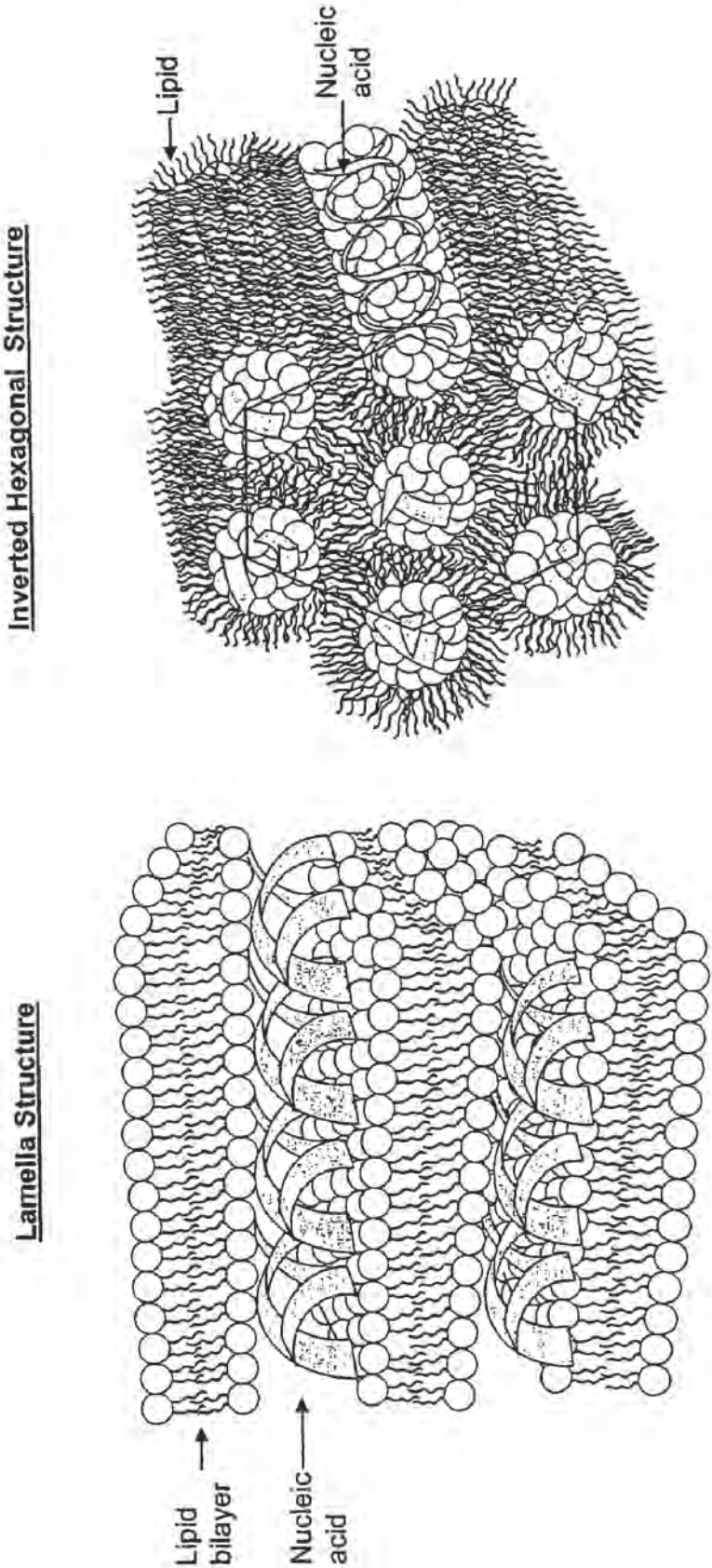
FIGURE 8

Lipid Composition in L051

CLinDMA

DSPC

Cholesterol

2KPEG-DMG
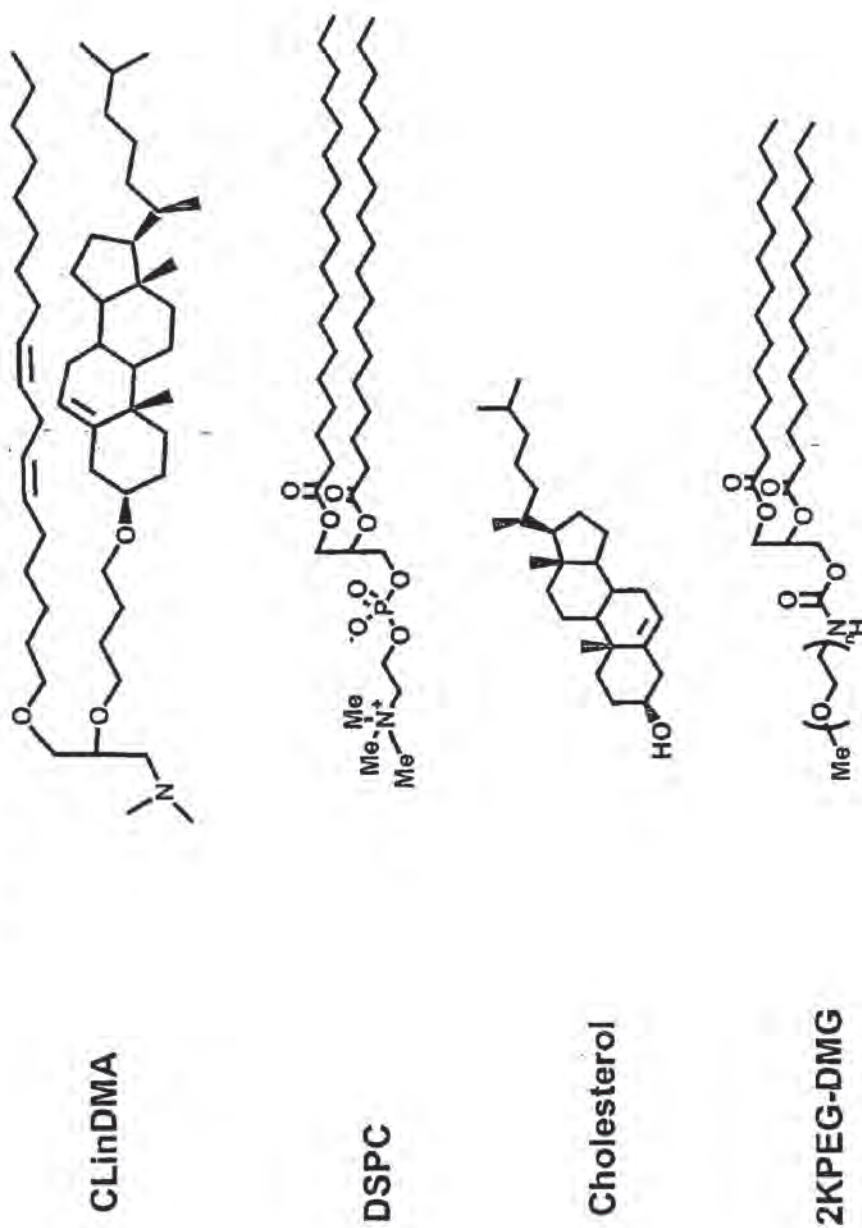
FIGURE 9
Lipid Structures in L073
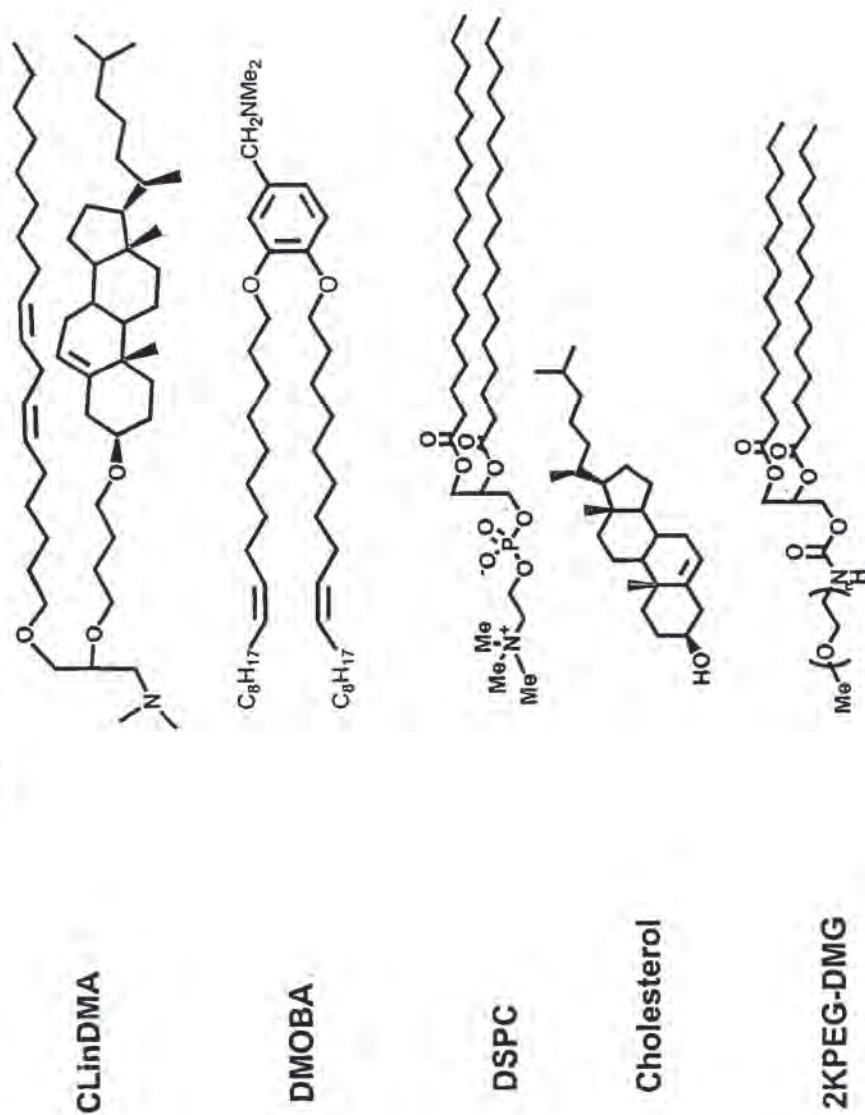

CLinDMA

DMOBA

DSPC

Cholesterol

2KPEG-DMG

FIGURE 10

Lipid Composition in L069
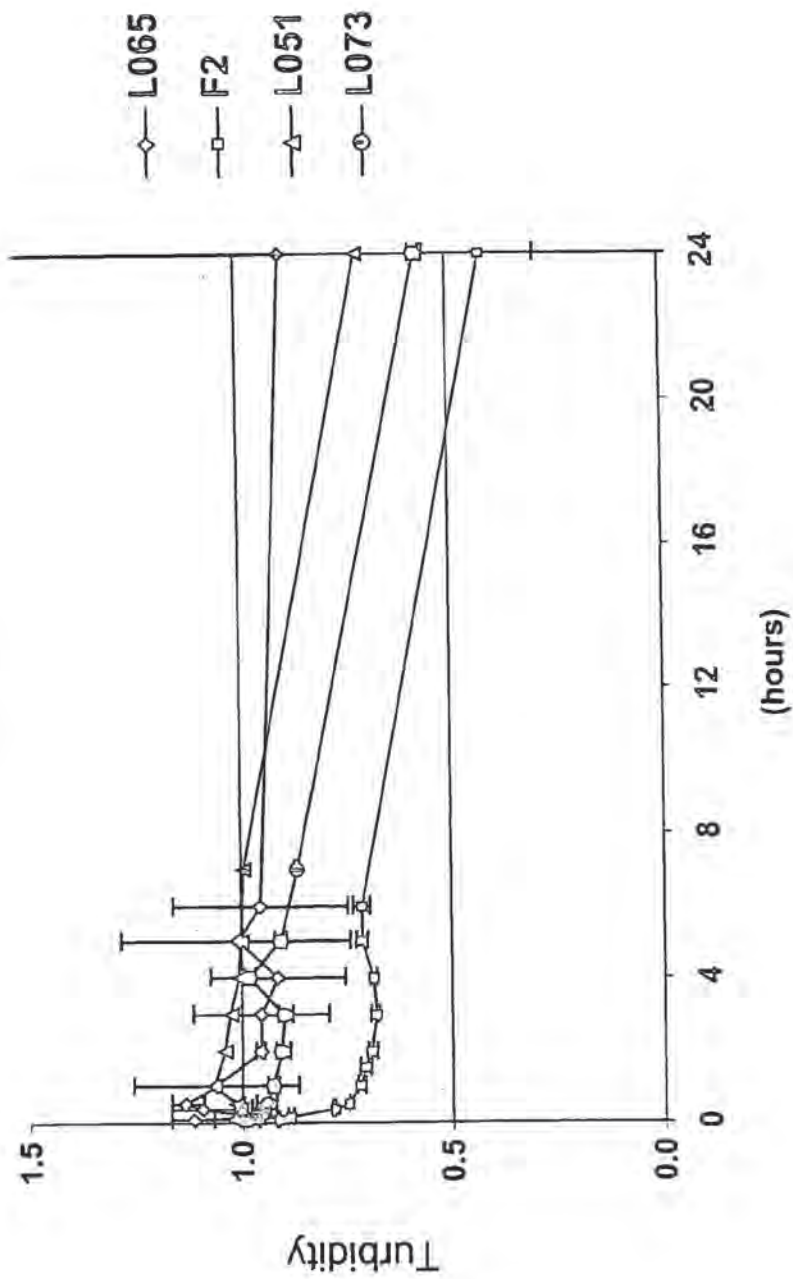

CLinDMA

DSPC
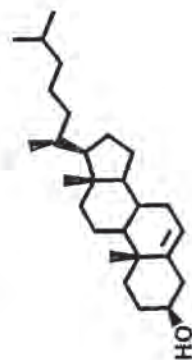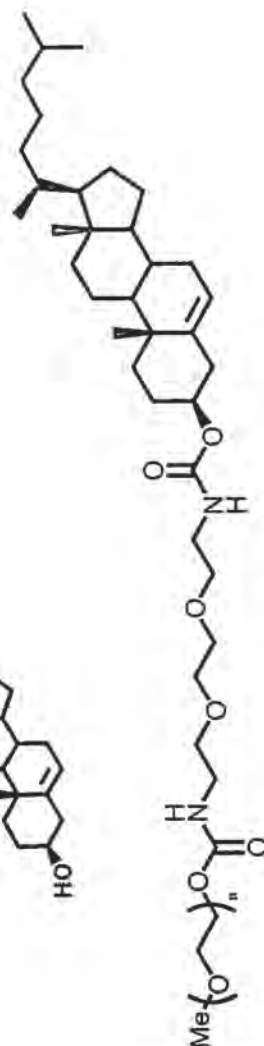
Cholesterol

Cholesterol-PEG

FIGURE 11

FIGURE 12

pH-dependent phase transition at 30min

FIGURE 13
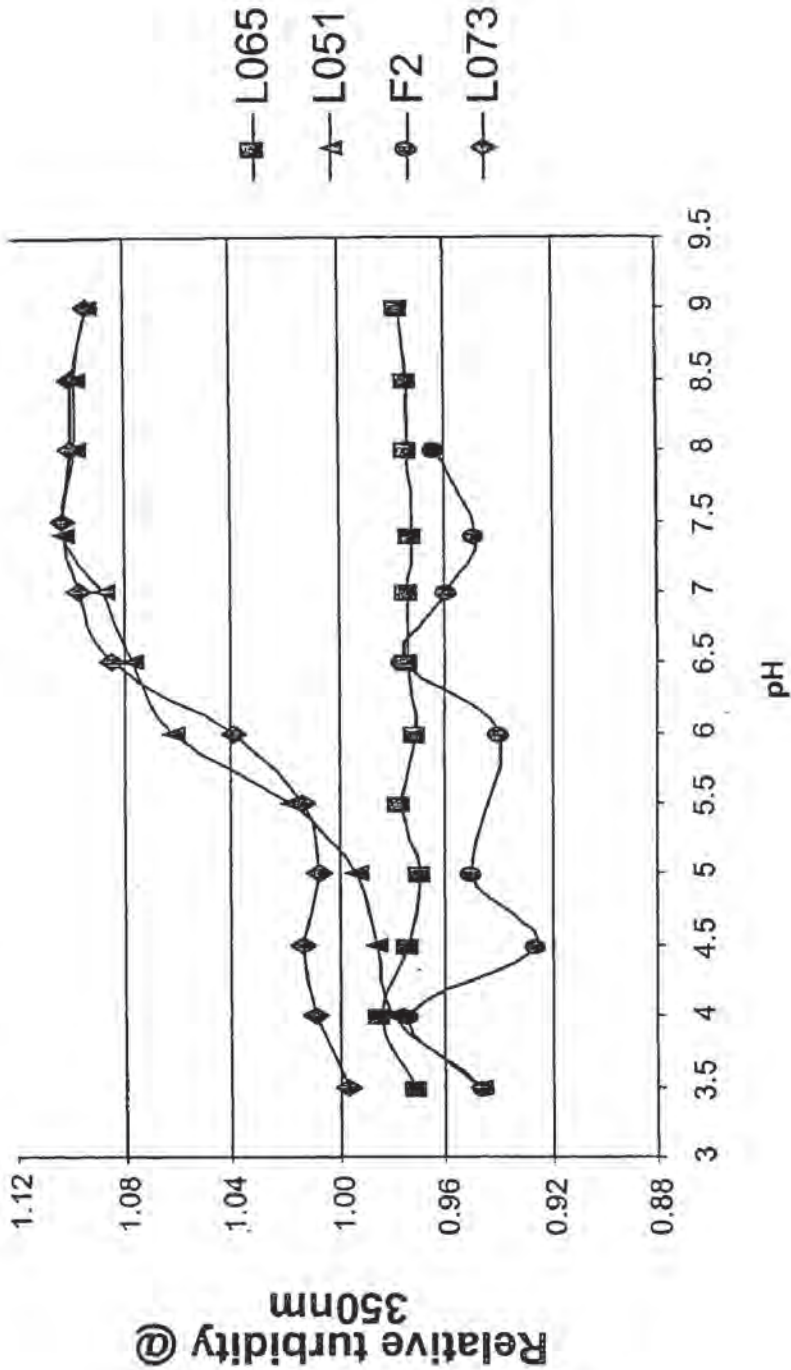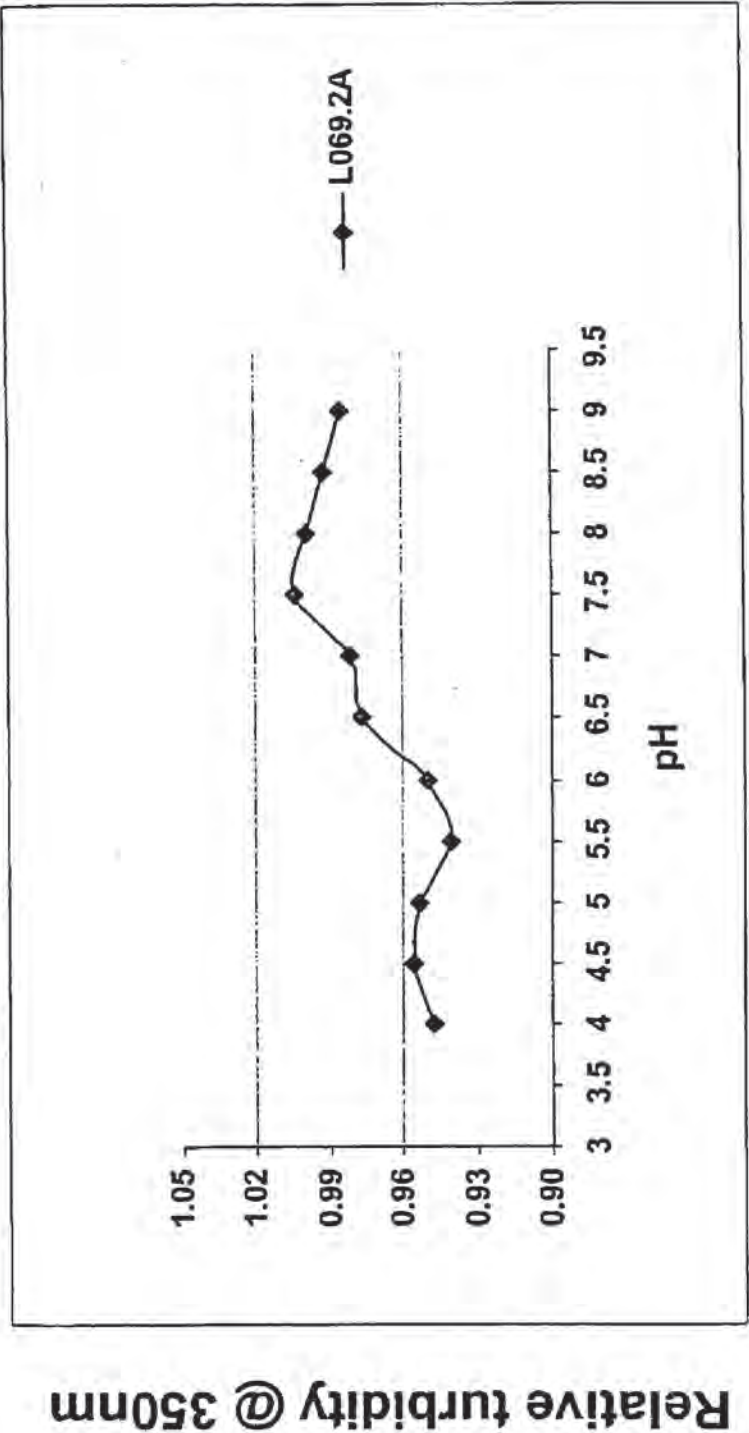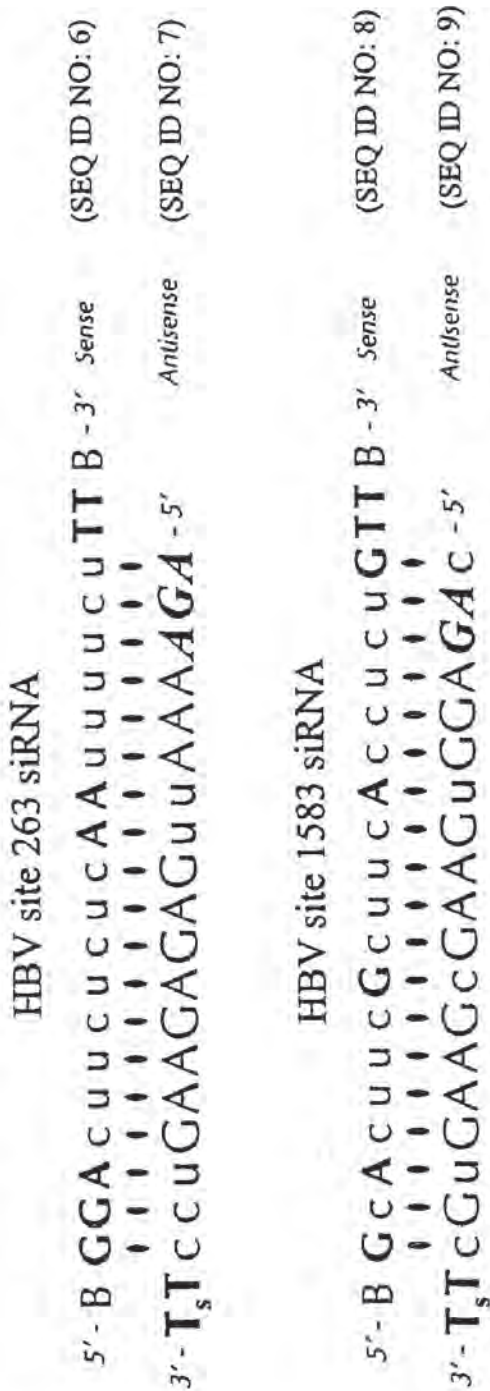
pH-dependent phase transition at 30min

FIGURE 14

HBV site 263 siRNA

5' - B GGAcucucucAAuuuucuTT B - 3' *Sense*   (SEQ ID NO: 6)

3' - T$_s$Tc c U GAAGAGAGu u AAA*AGA* - 5' *Antisense*   (SEQ ID NO: 7)

HBV site 1583 siRNA

5' - B GcAcuucGcuucAccucuGTT B - 3' *Sense*   (SEQ ID NO: 8)

3' - T$_s$Tc CuGAAGcGAAGuGGAGAc - 5' *Antisense*   (SEQ ID NO: 9)

AGT = *deoxy A, G & T*
AGCU = *ribo A, G, C, U*
AG = *2'-O-methyl A & G*
cu = *2'-fluoro C & U*
B   = *3',5' inverted deoxy abasic*
$_s$ = *phosphorothioate*

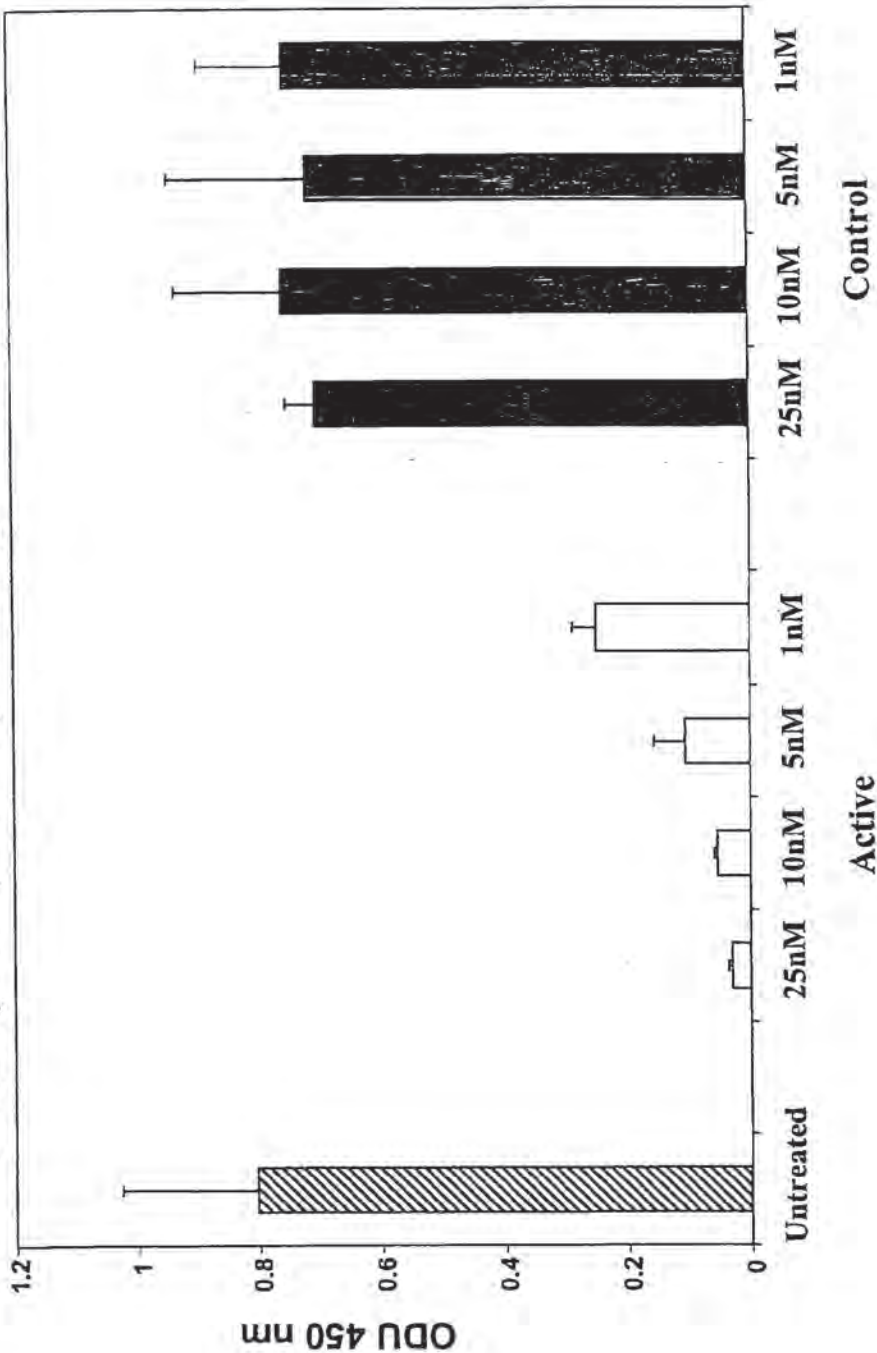FIGURE 15: In vitro Analysis of siNA Formulation L051 Activity in HBV Expressing Hep G2 Cells

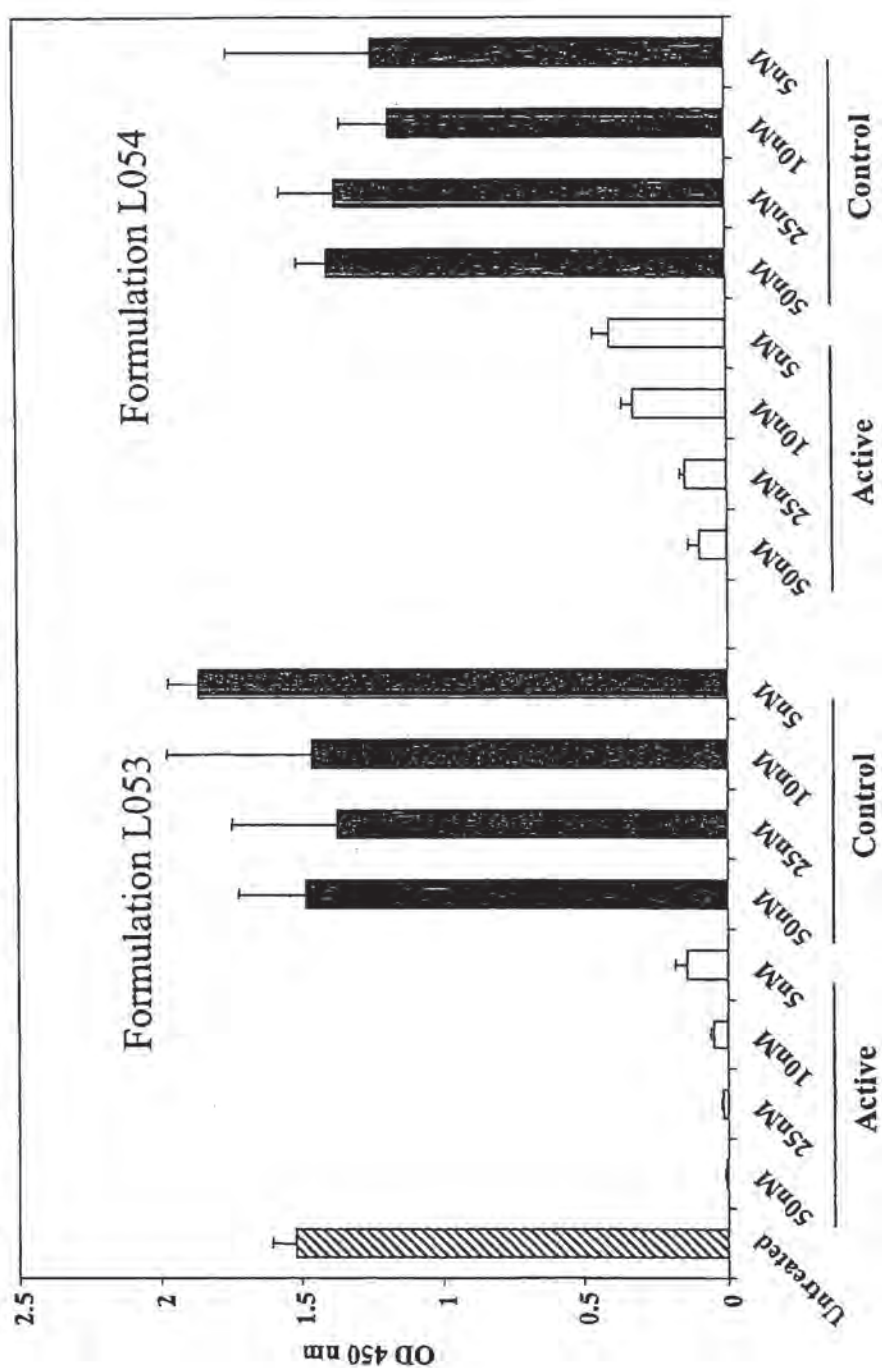FIGURE 16: In vitro Analysis of siNA Formulation L053 and L054 activity in HBV Expressing Hep G2 Cells

FIGURE 17

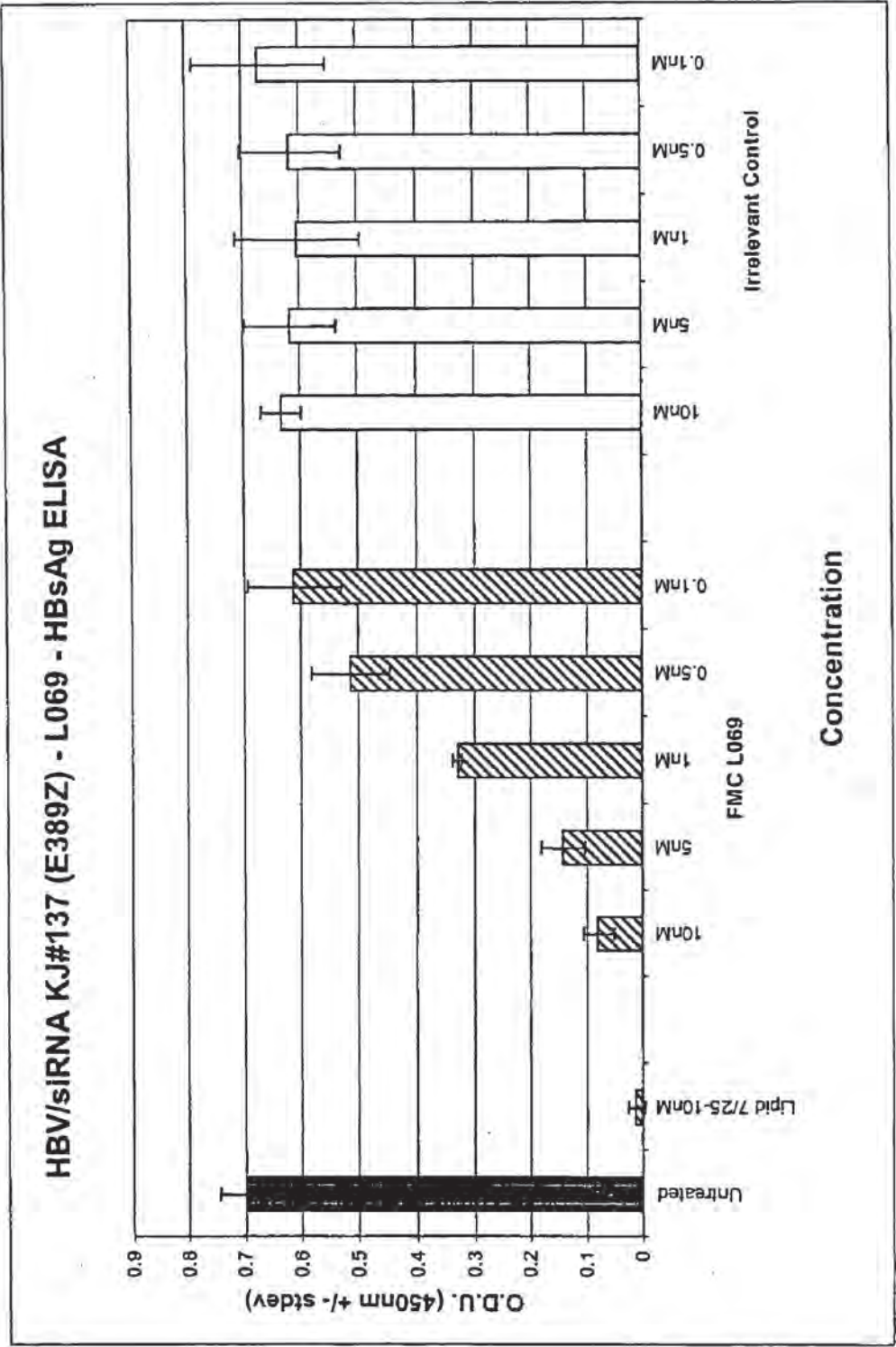HBV/siRNA KJ#137 (E389Z) - L069 - HBsAg ELISA

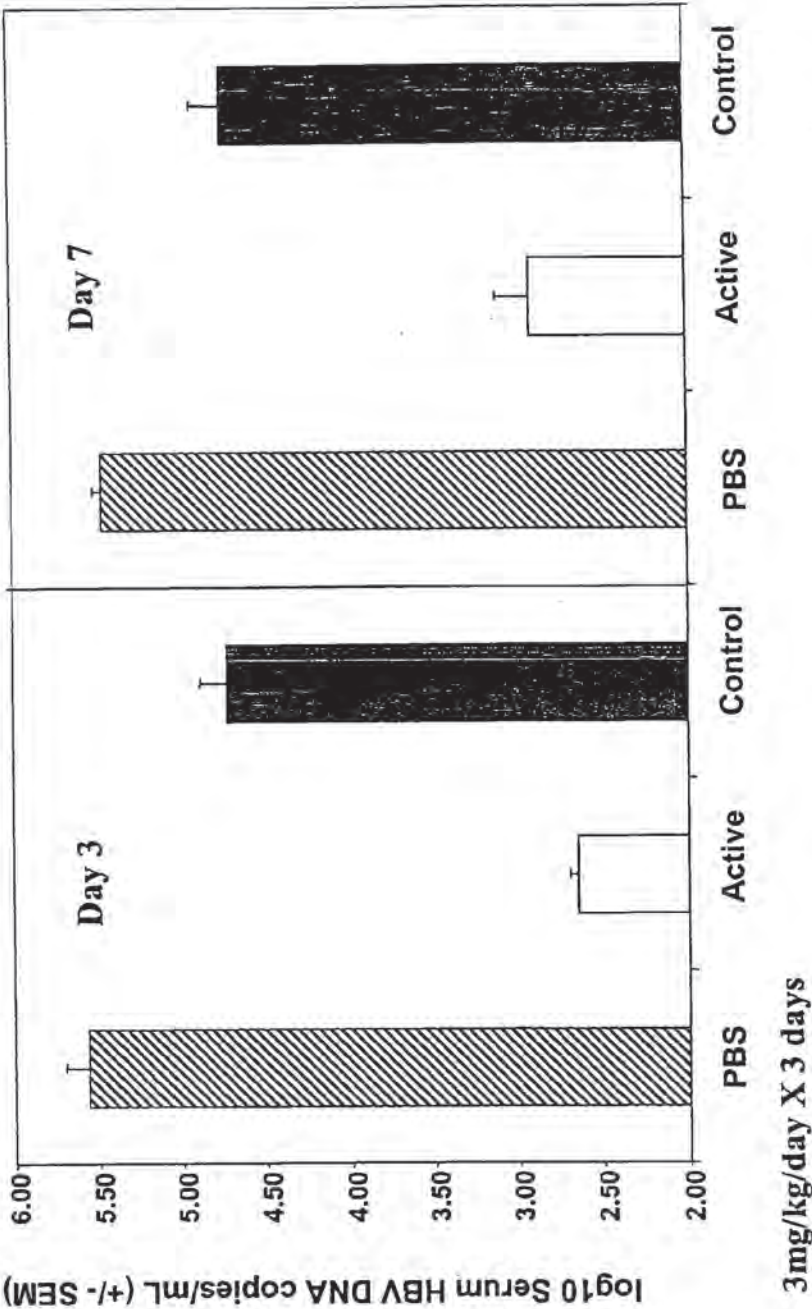FIGURE 18: Activity of siNA Formulation L051 compositions in HBV Mouse Model, Serum HBV DNA

FIGURE 19: Activity of siNA siNA Formulation L051 compositions in HBV Mouse Model, Serum HBsAg
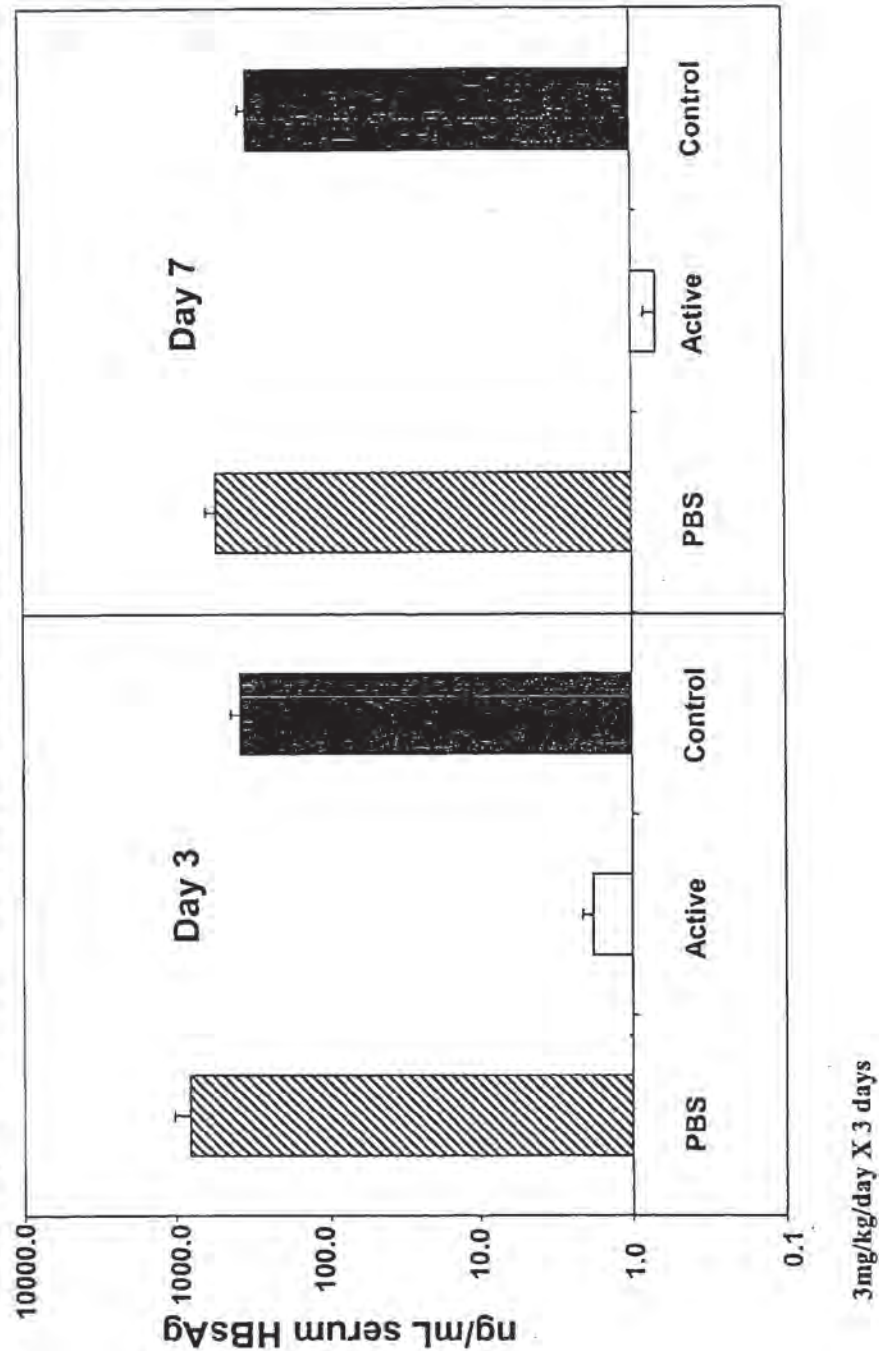
JA001105

FIGURE 20: In Vitro Analysis of siNA Formulation L051 Activity in HCV Replicon System
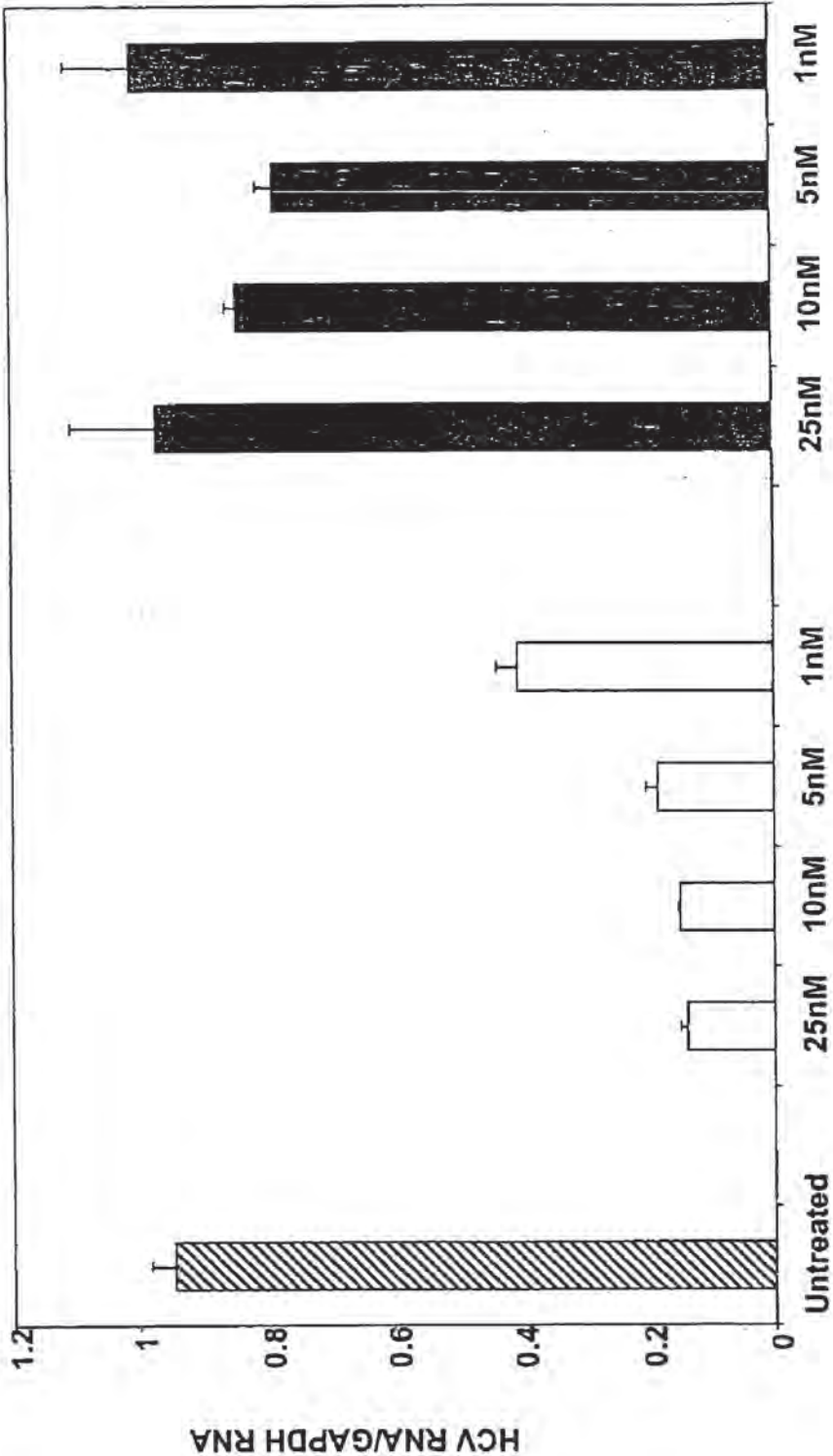
FIGURE 21: In Vitro Analysis of siNA Formulation L053 and L054 Activity in HCV Replicon System
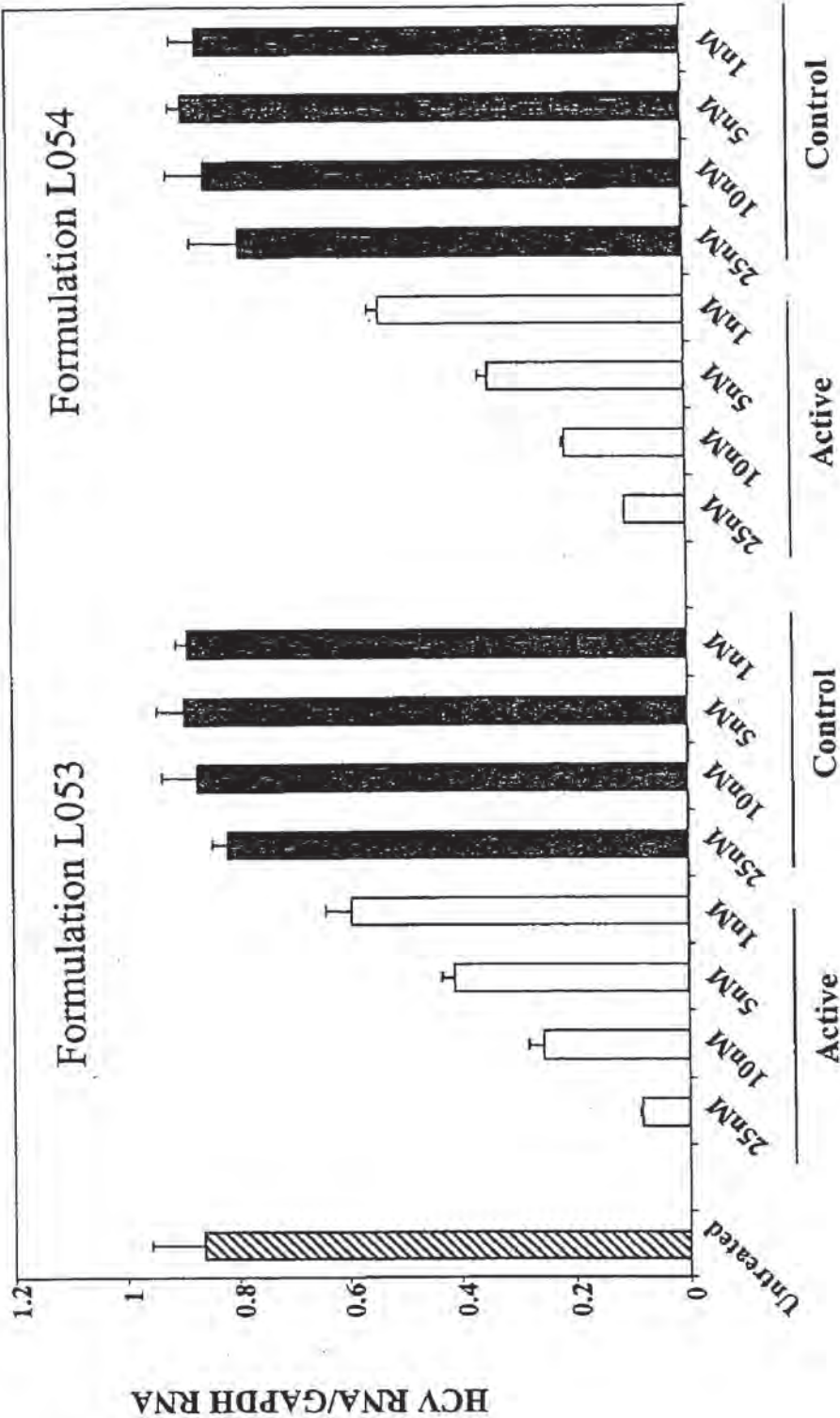
FIGURE 22

Concentration of siRNA in the Mouse Lung after IT Administration (20 μg)
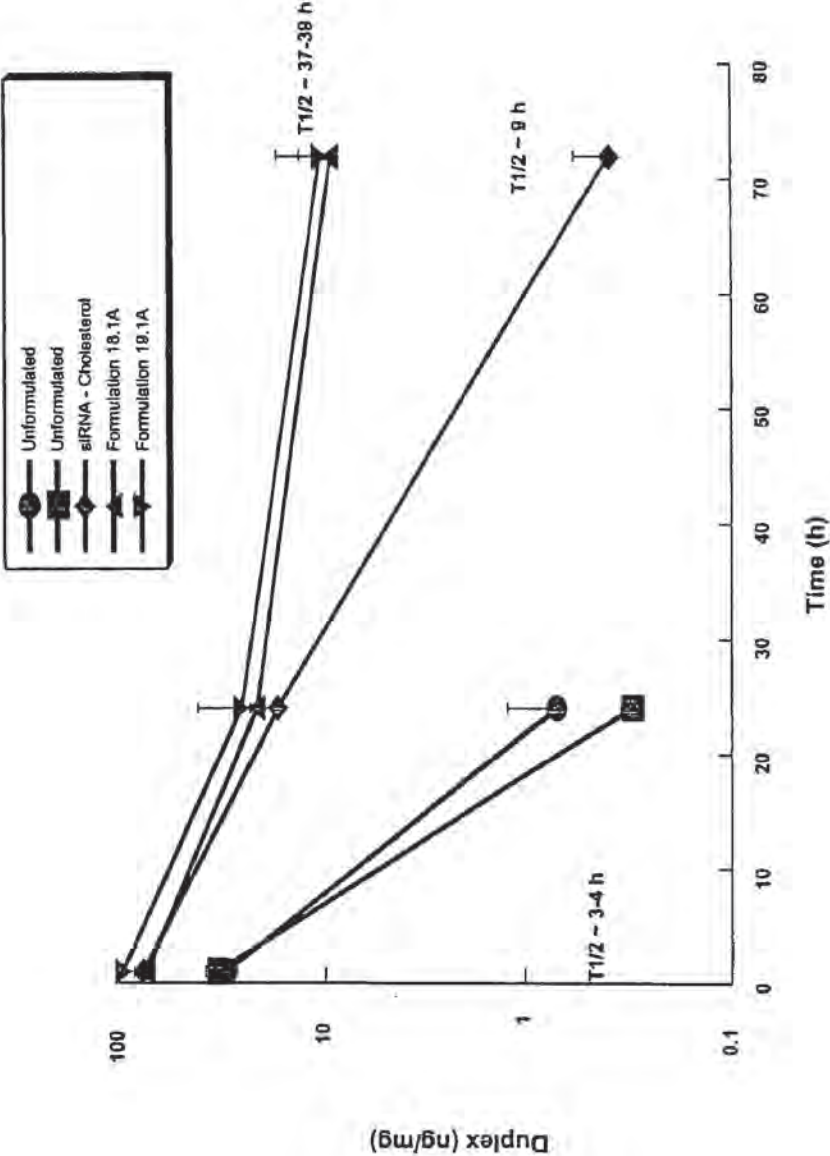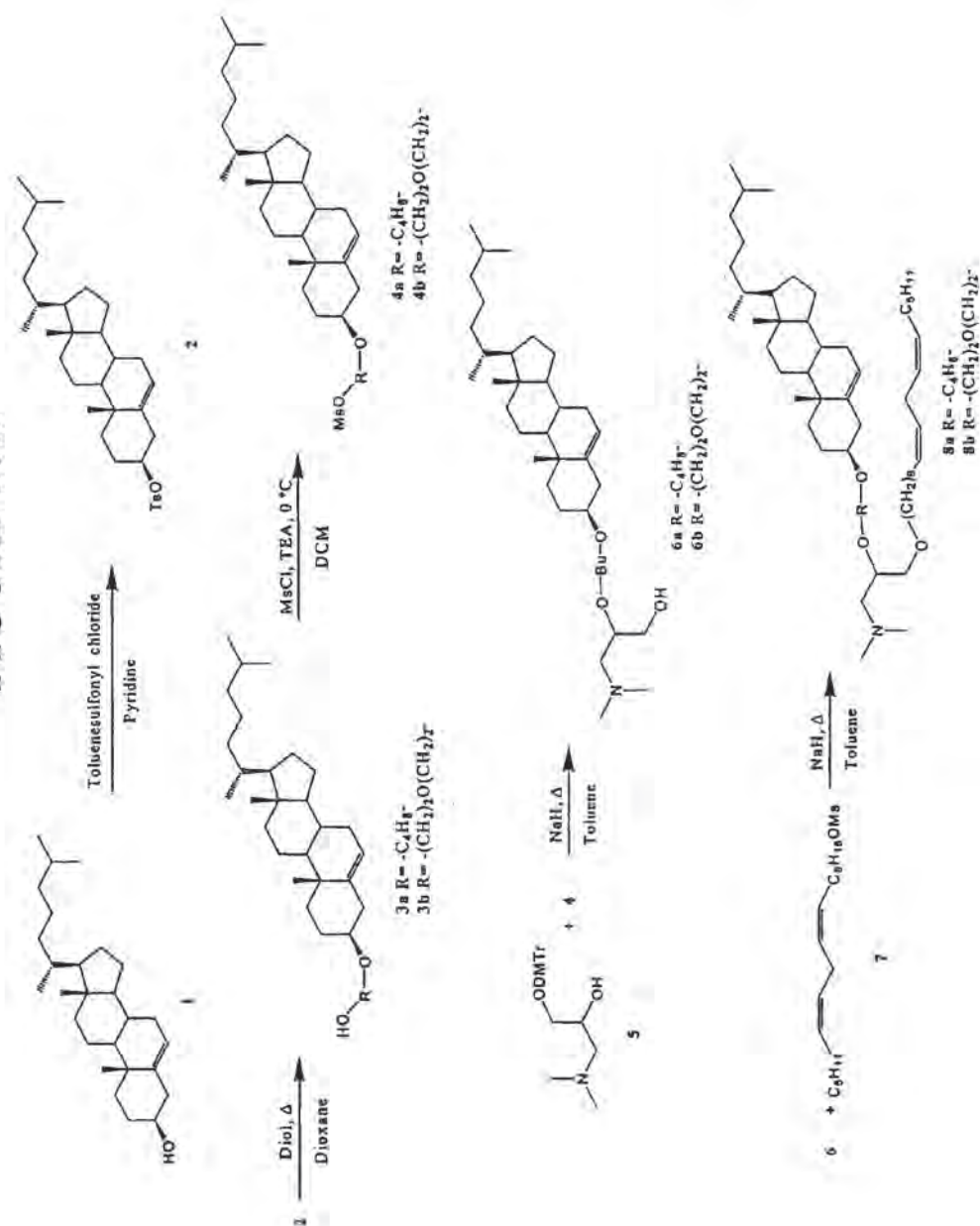
FIGURE 23A

Figure 23B

FIGURE 24

Cholesterol-PEG

DMB-Peg
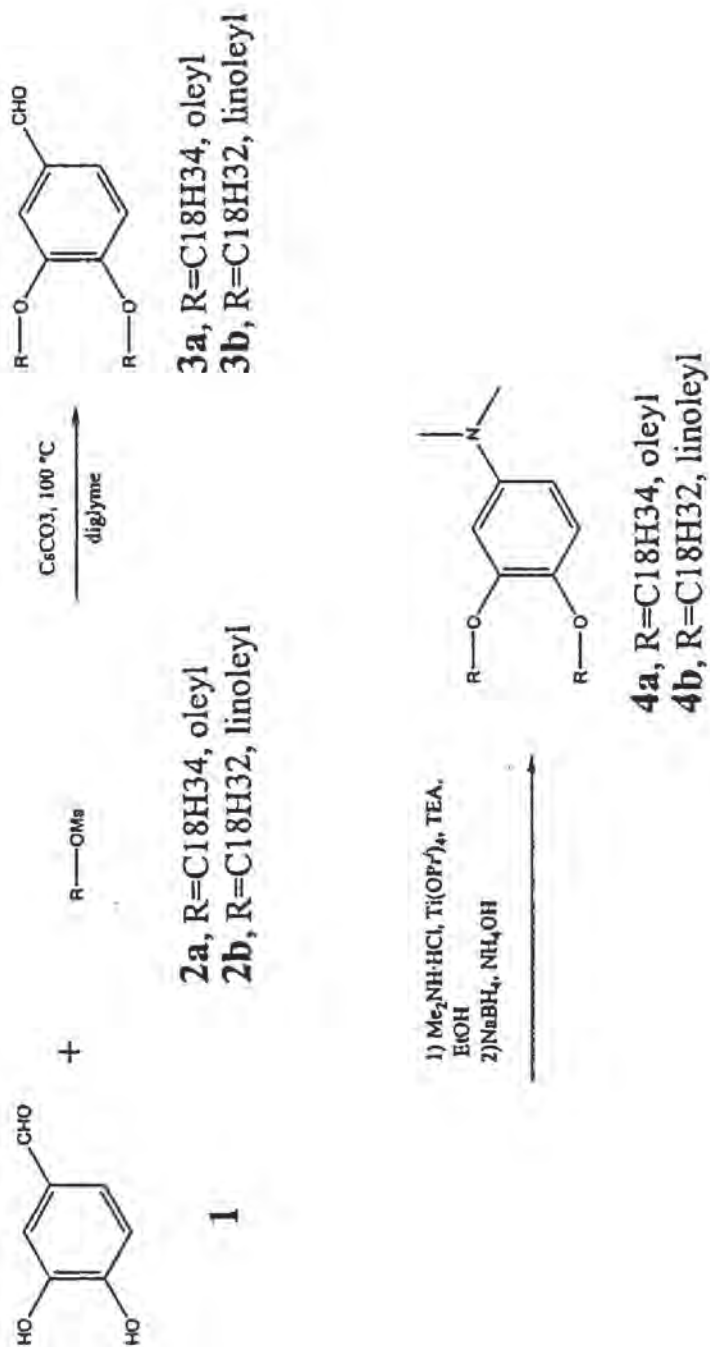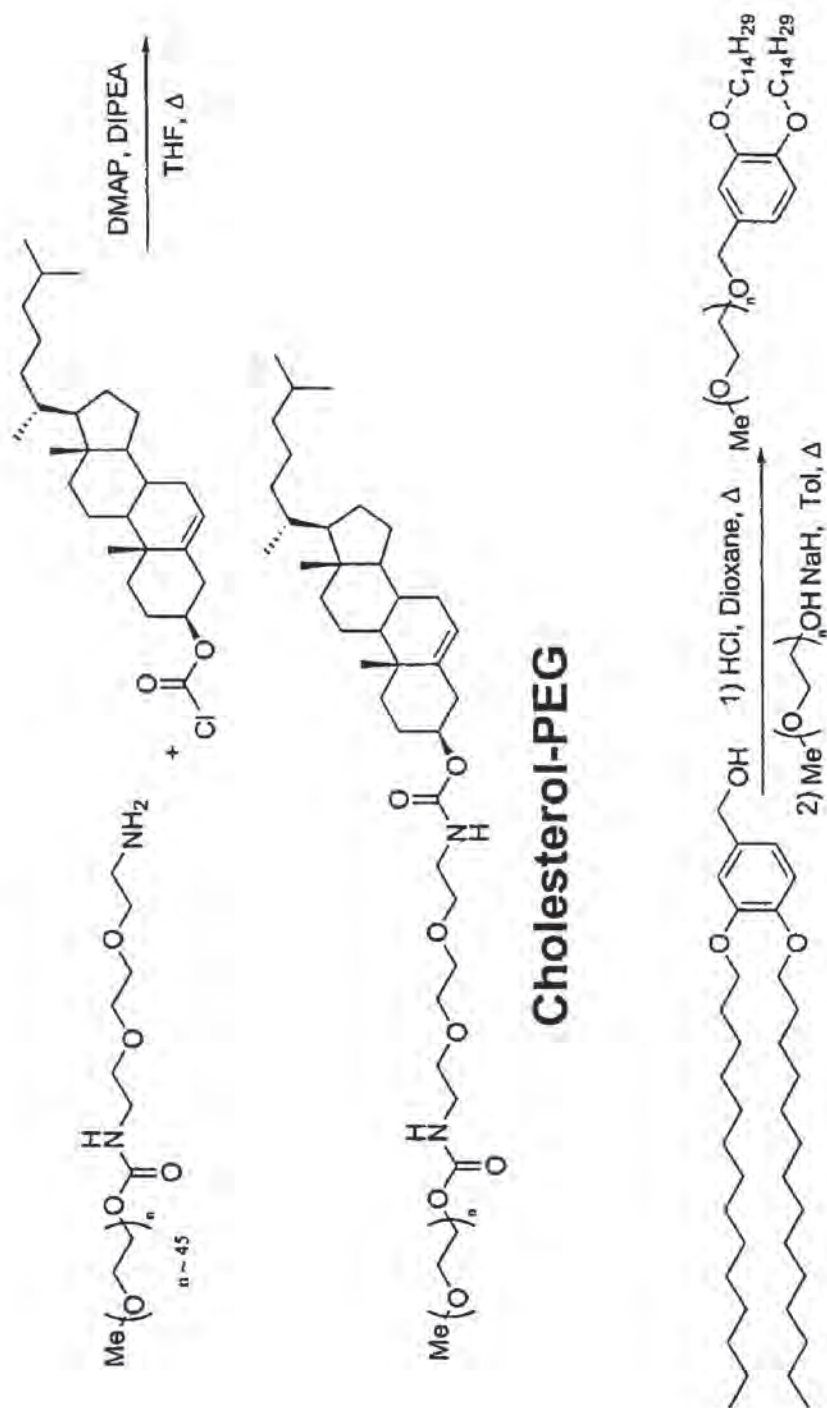
FIGURE 25

Lipid Composition in LNP 083
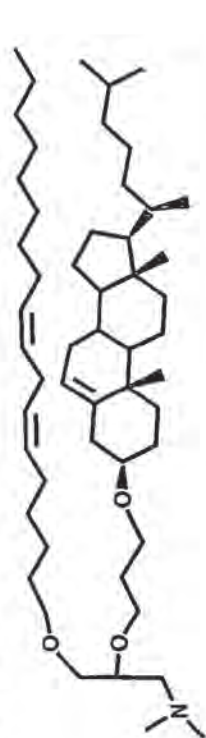
pCLinDMA (48%)

DSPC (40%)
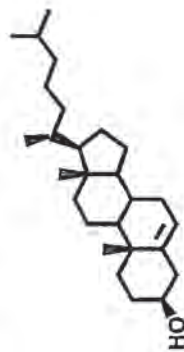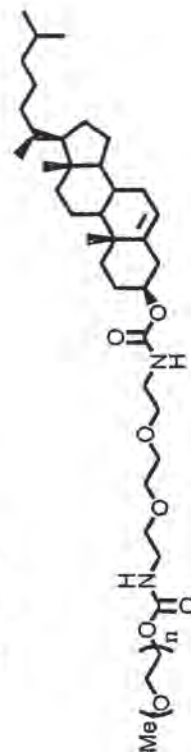
Cholesterol (10%)

2KPEG-Chol (2%)

FIGURE 26

Lipid Composition in LNP 077

eCLinDMA (48%)

DSPC (40%)

Cholesterol(10%)

2KPEG-Chol (2%)
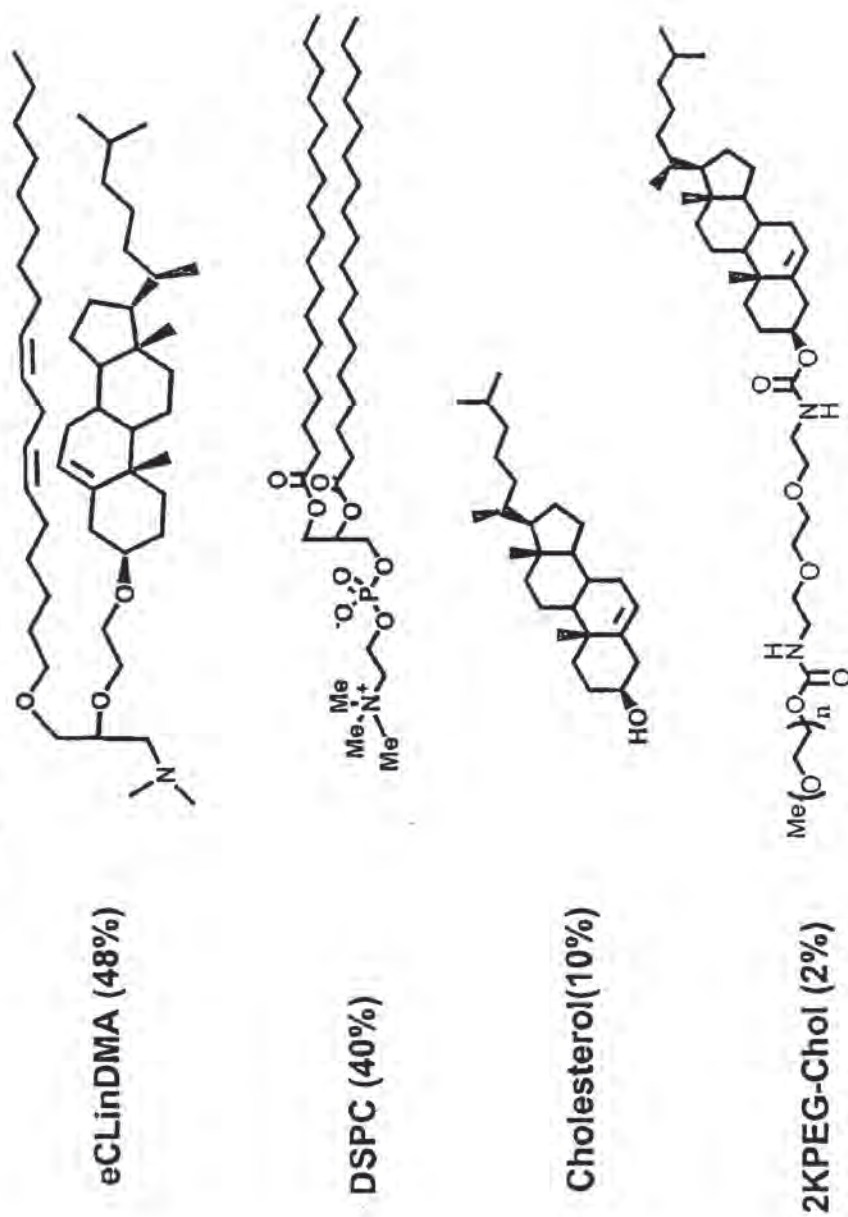
FIGURE 27

Lipid Composition in LNP 080

eCLinDMA (48%)

DSPC (40%)

Cholesterol(10%)
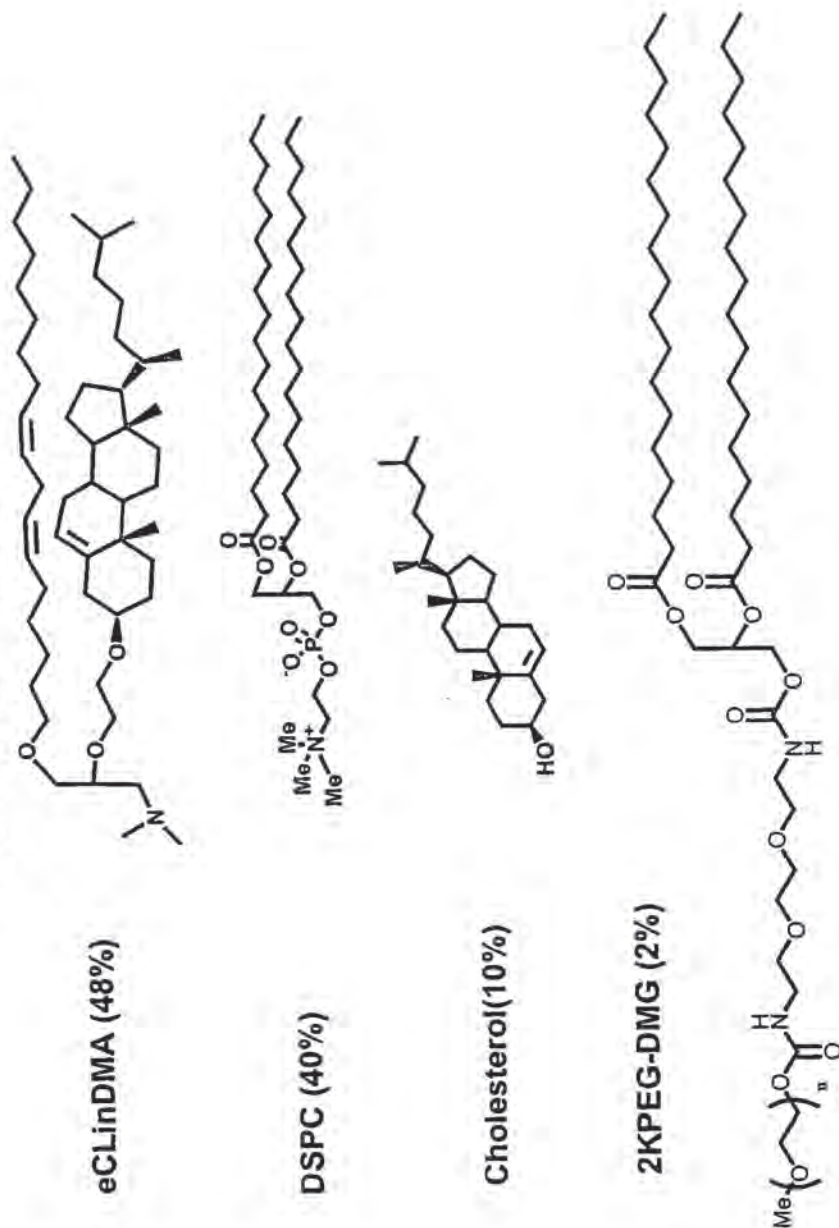
2KPEG-DMG (2%)

FIGURE 28

Lipid Composition in LNP 082
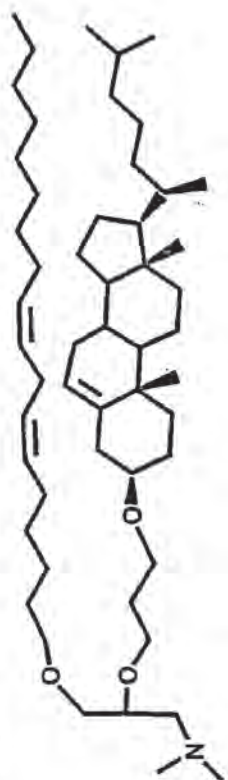
pCLinDMA (48%)

DSPC (40%)
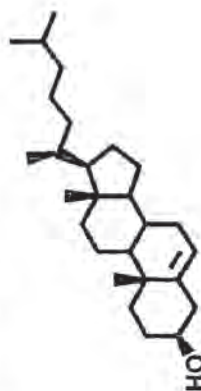
Cholesterol(10%)
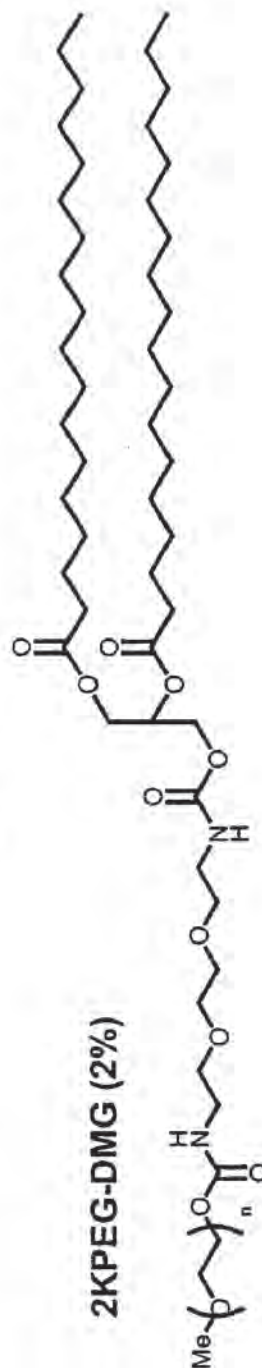
2KPEG-DMG (2%)

FIGURE 29

Serum HBV DNA in SCID mice: siRNA formulation comparison

3 mg/kg/d IV x 3 days; siRNA formulated HBV site 263 7/25
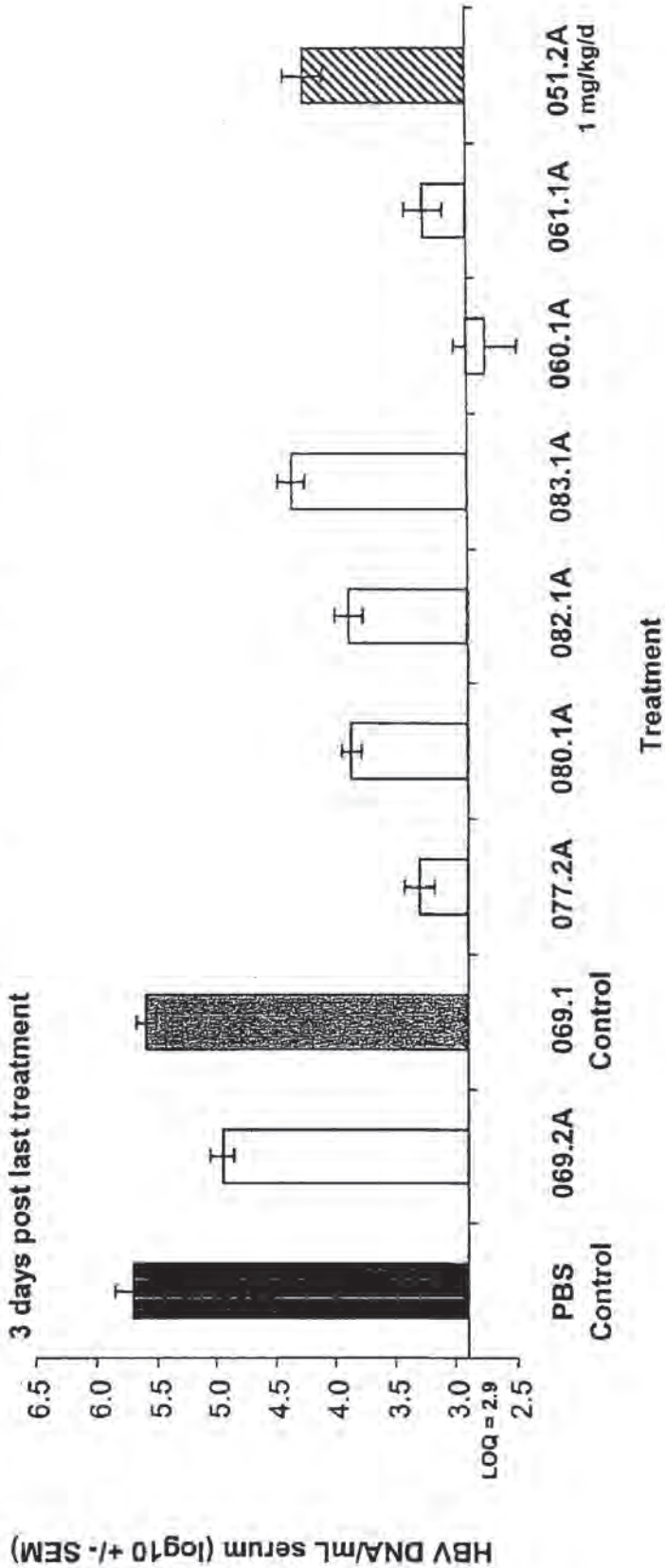
FIGURE 30

HBV/siRNA Sirna 083 and 084-Site 263 stab 7/25-HBsAg ELISA
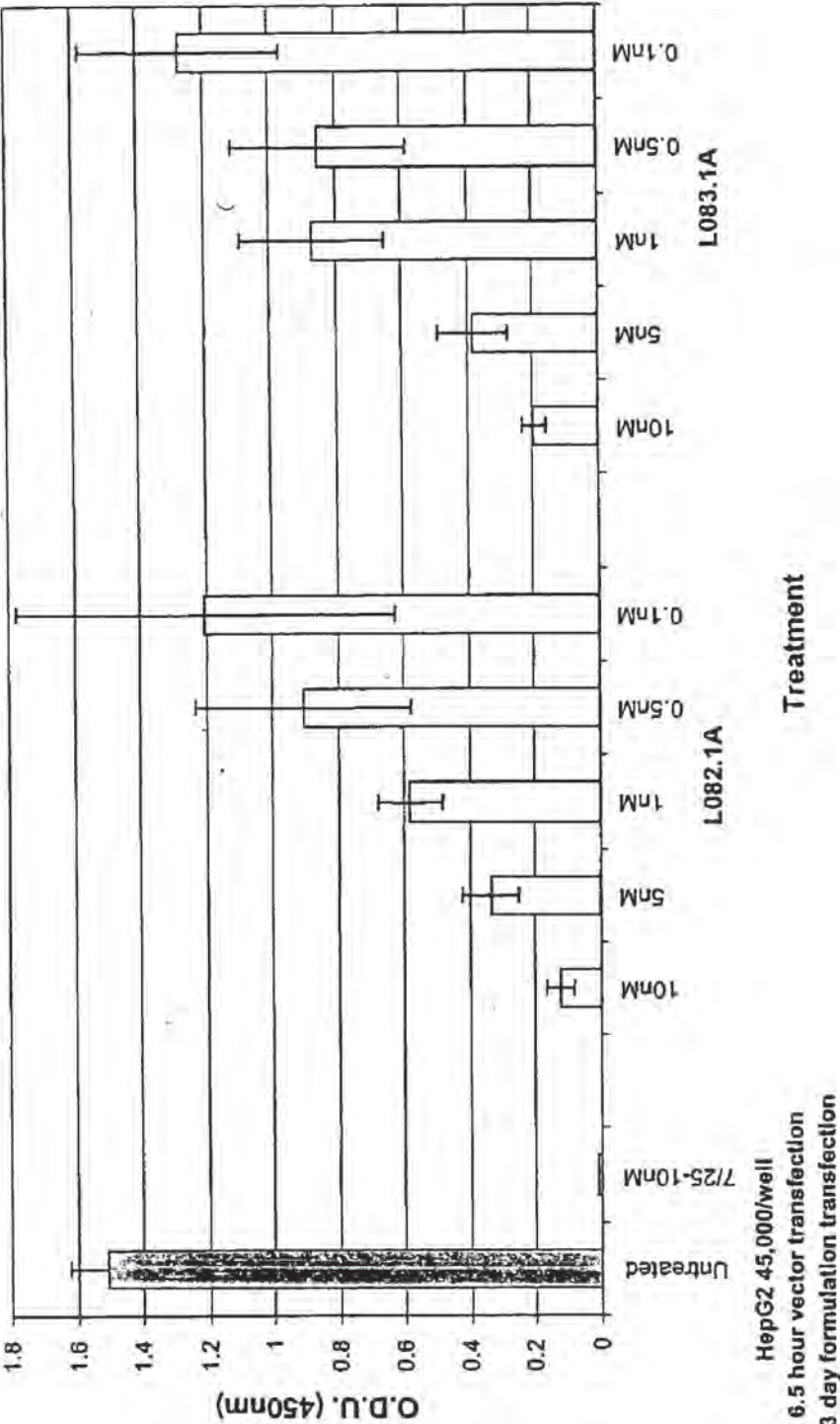
FIGURE 31

FIGURE 32

HBV/siRNA Sirna Formulation 080-HBsAg ELISA
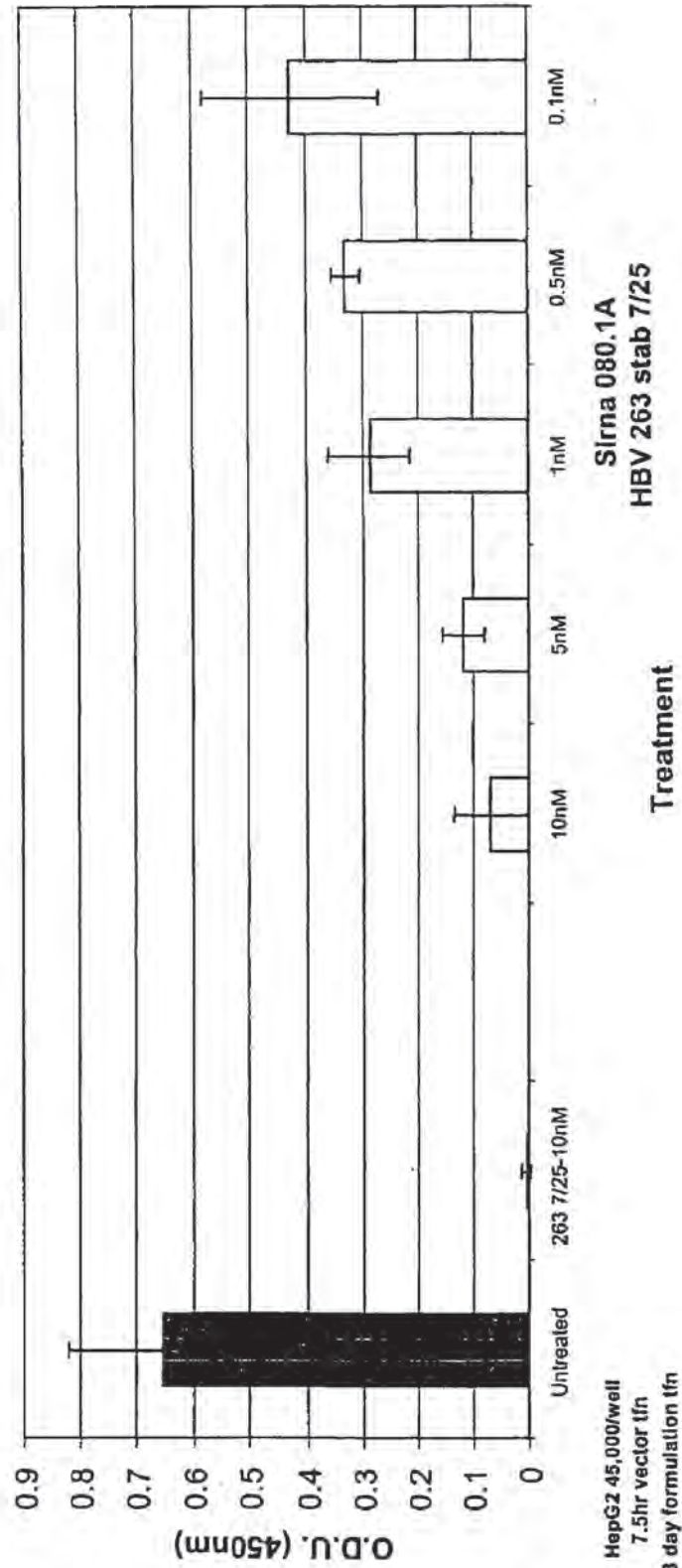
FIGURE 33

The serum stability of lipid nano particles
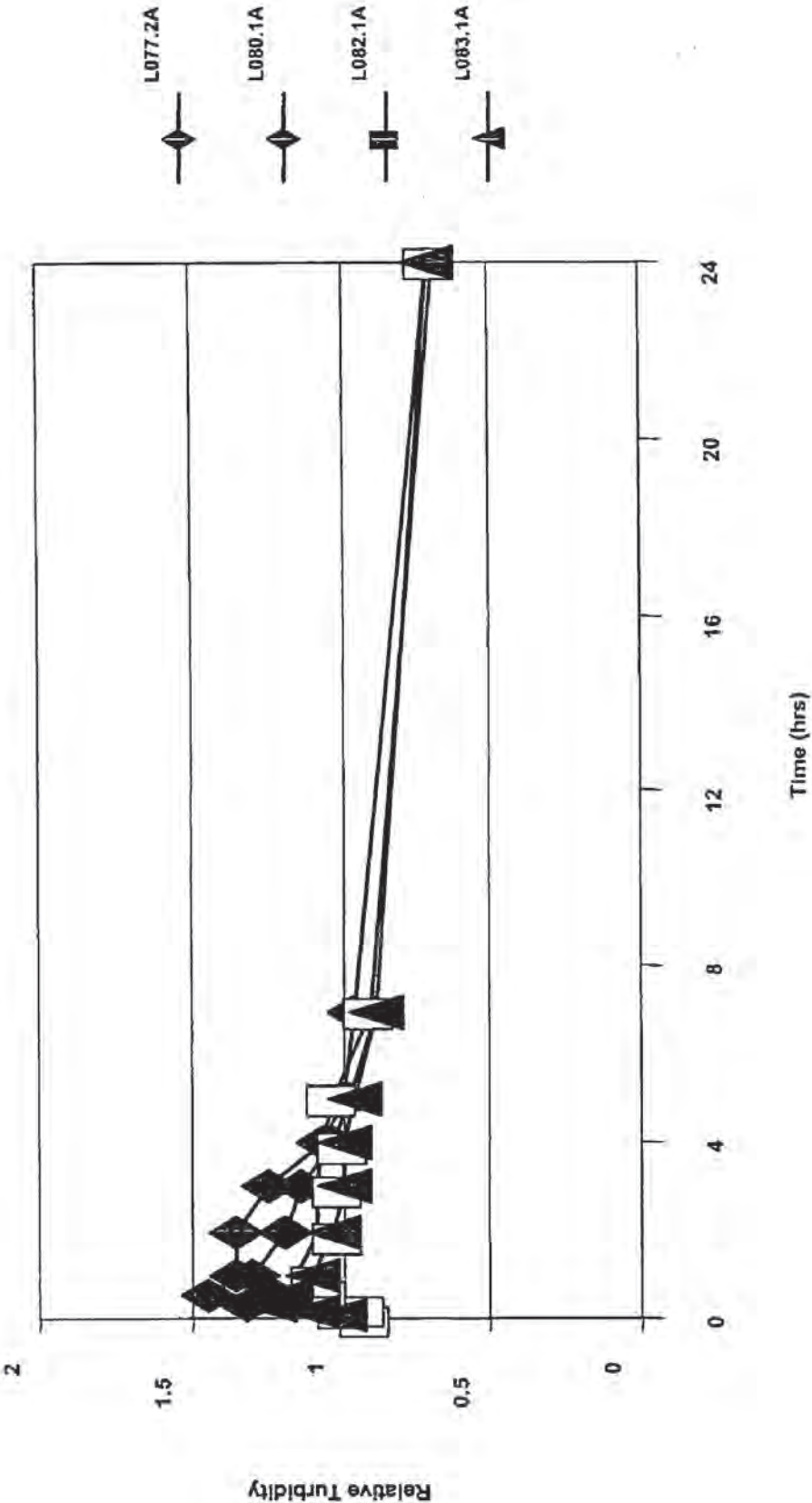
FIGURE 34

Phase transition of lipid nano particles
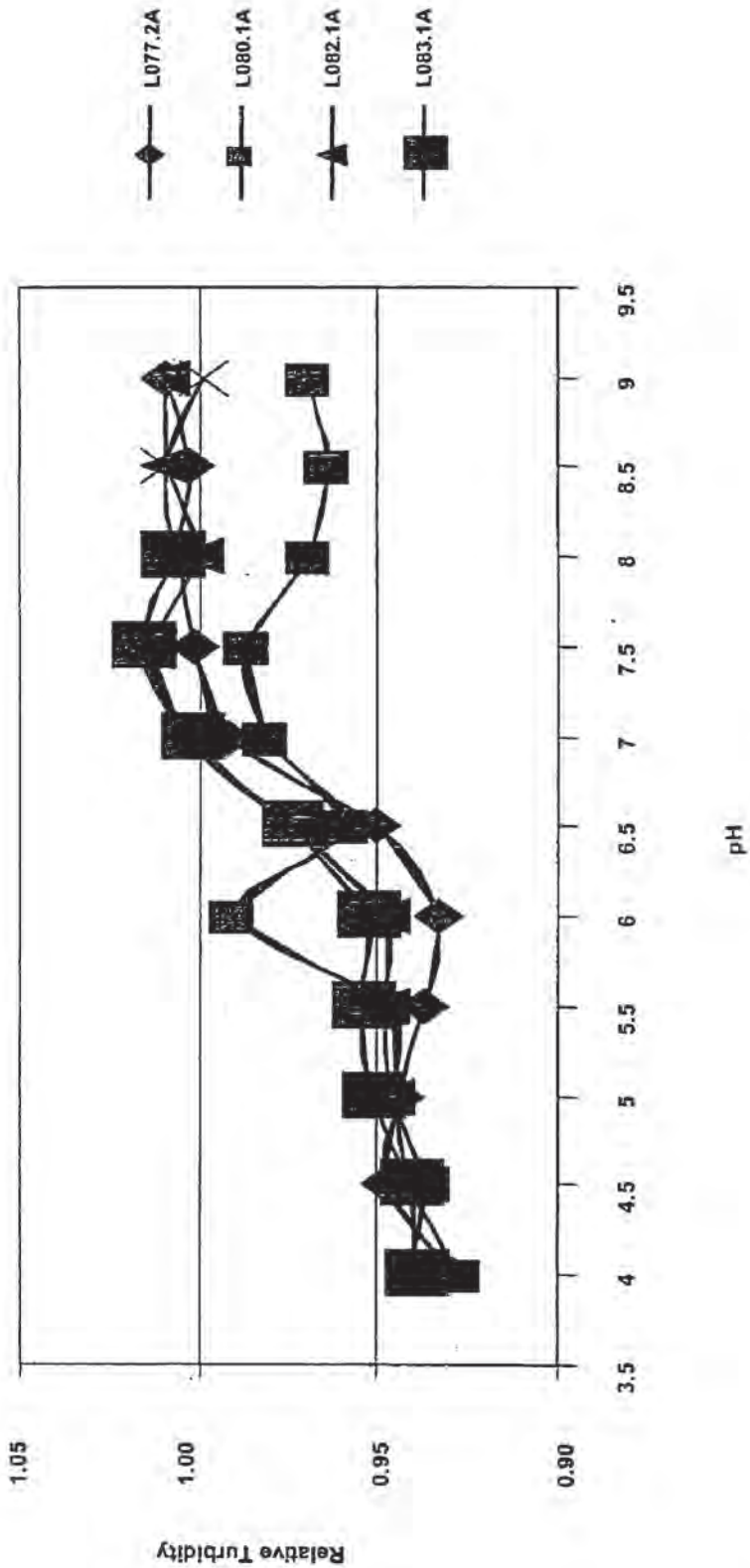
Figure 35
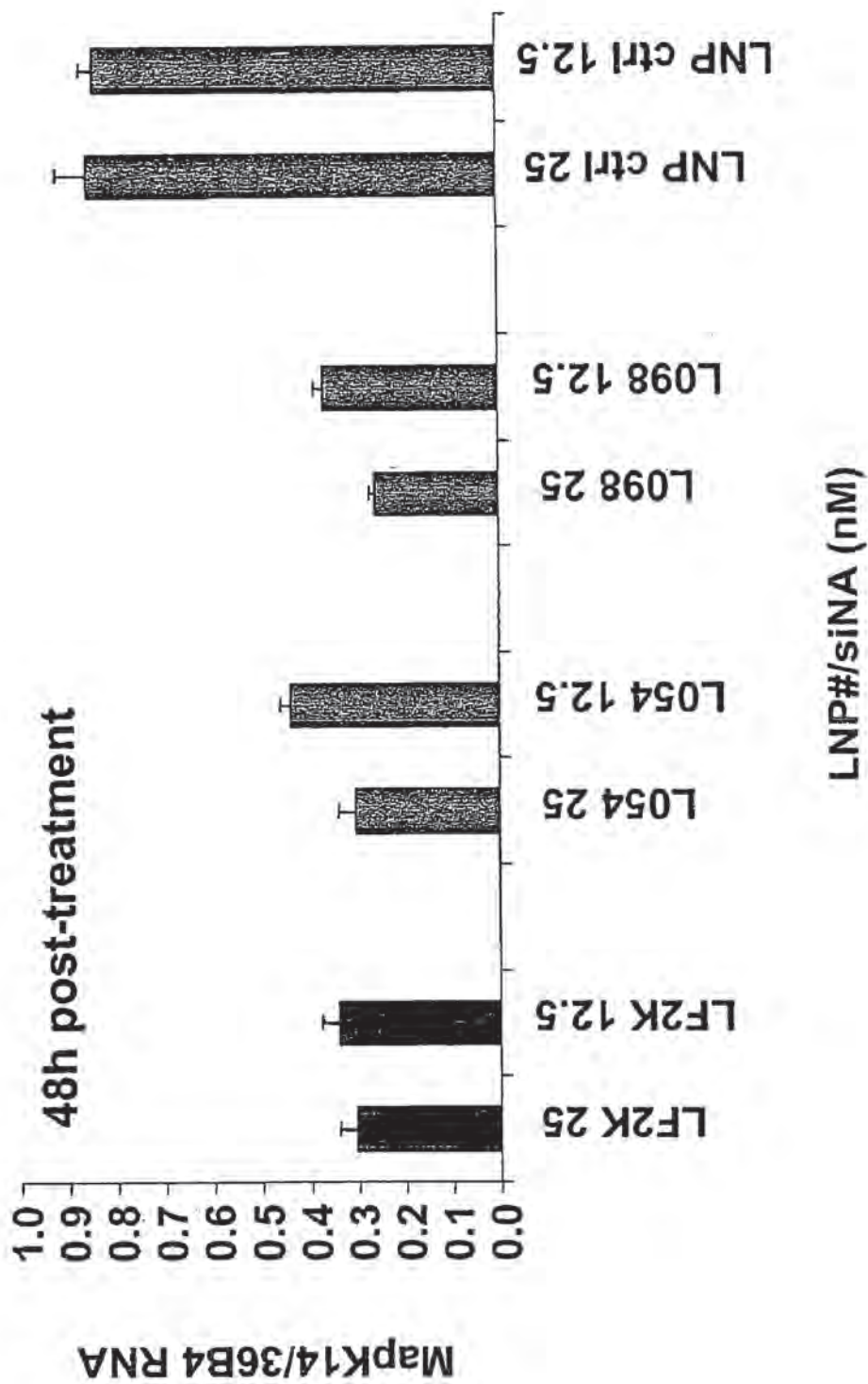
Figure 36
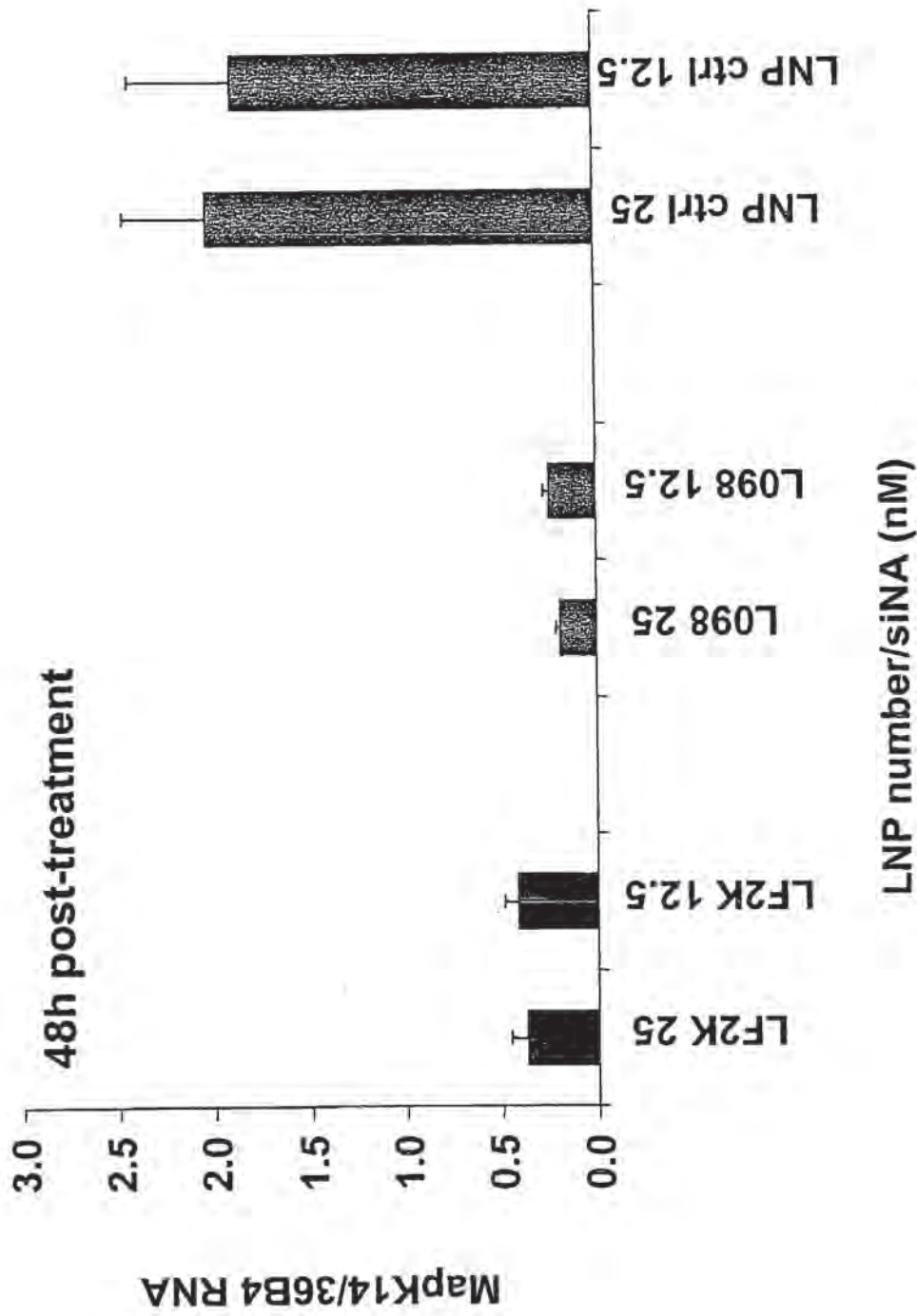
Figure 37
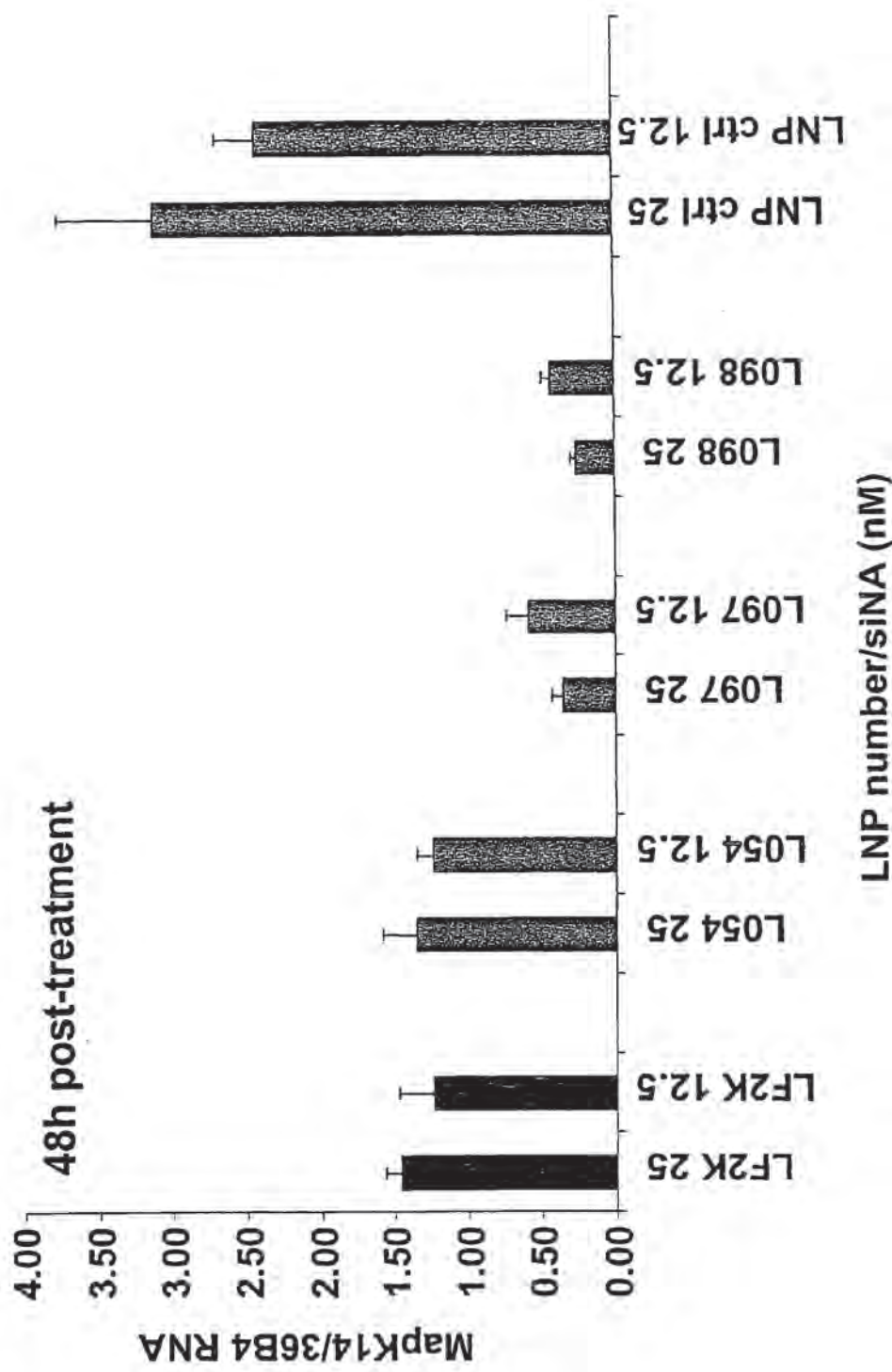
Figure 38
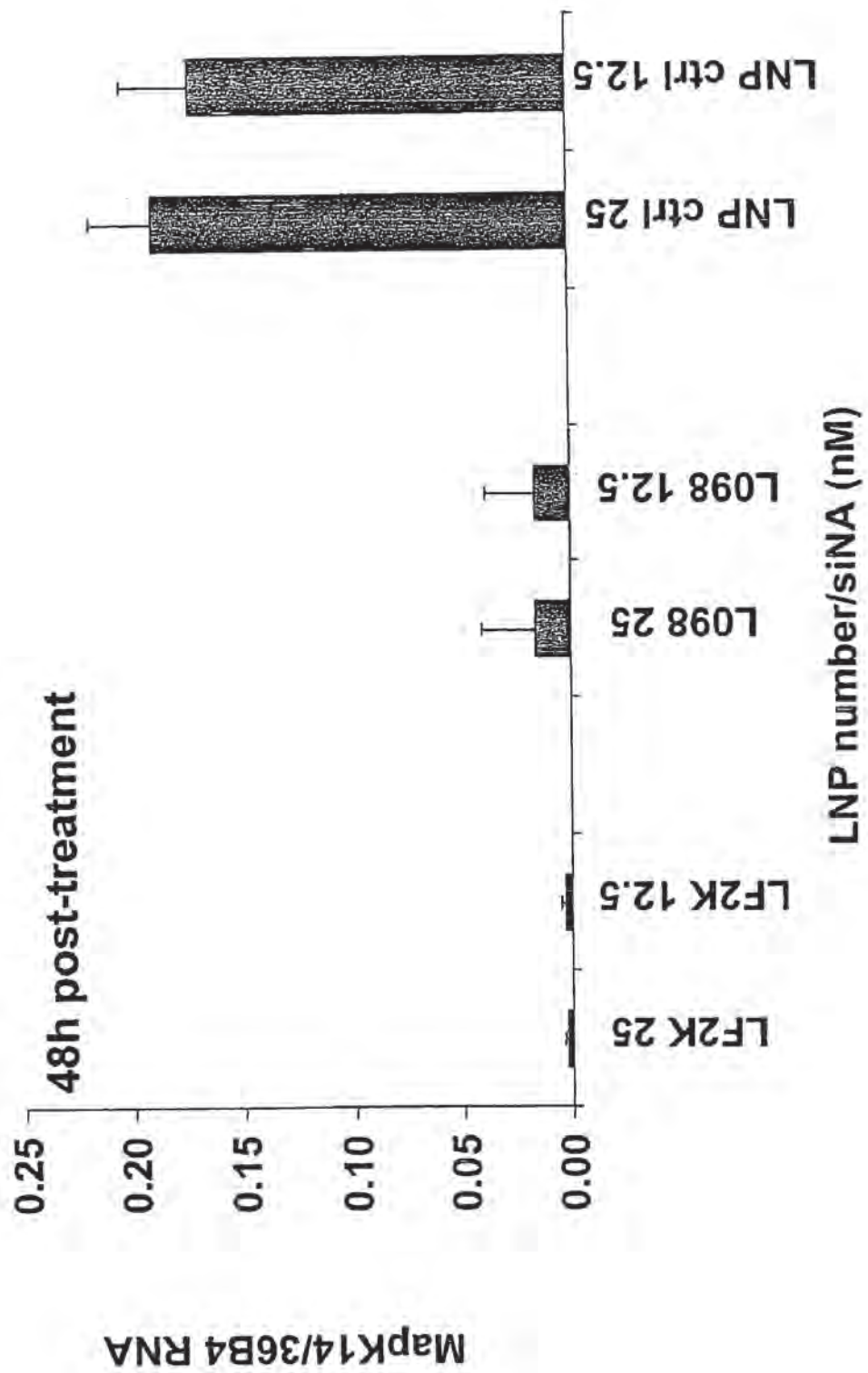
Figure 39
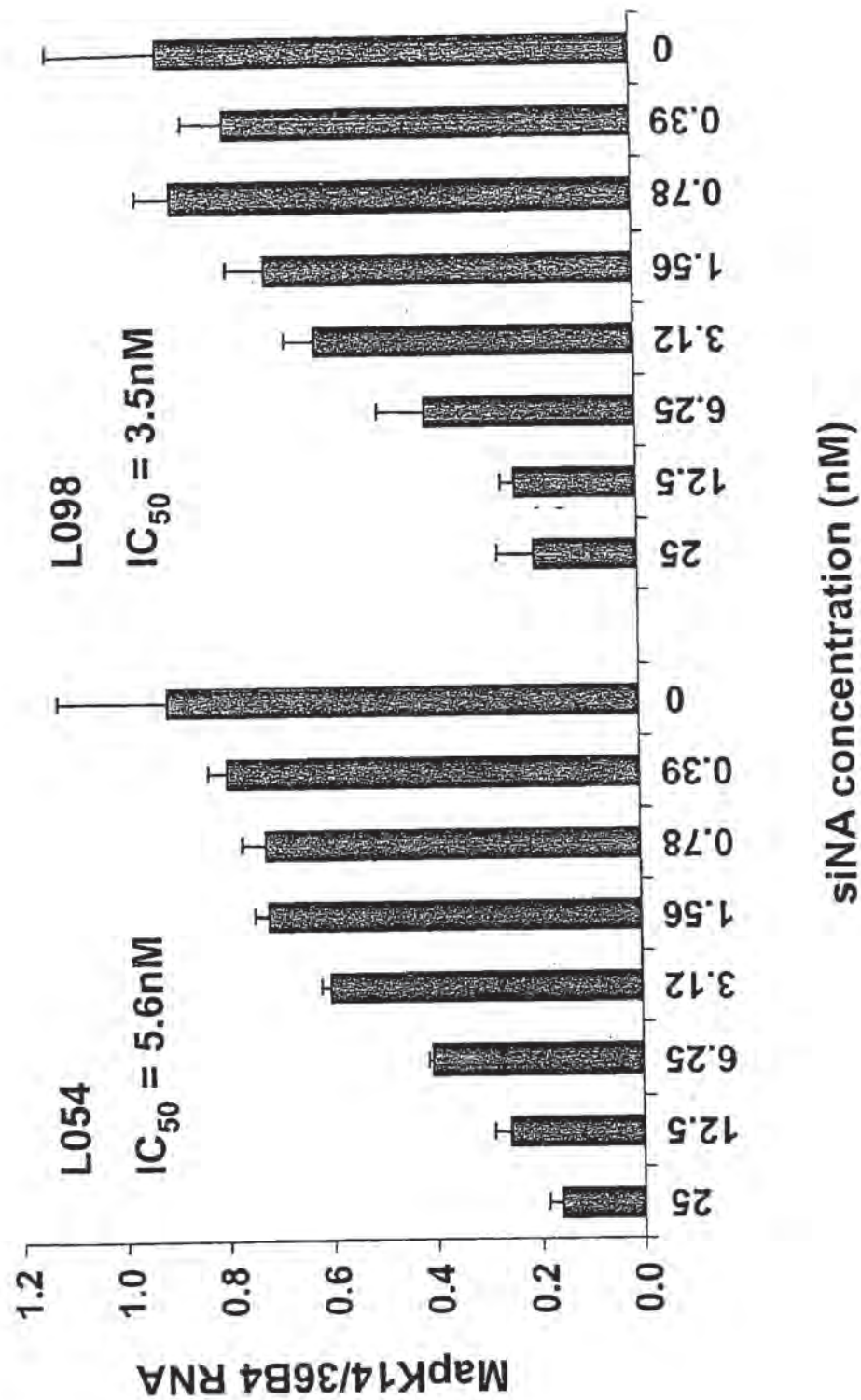
Figure 40
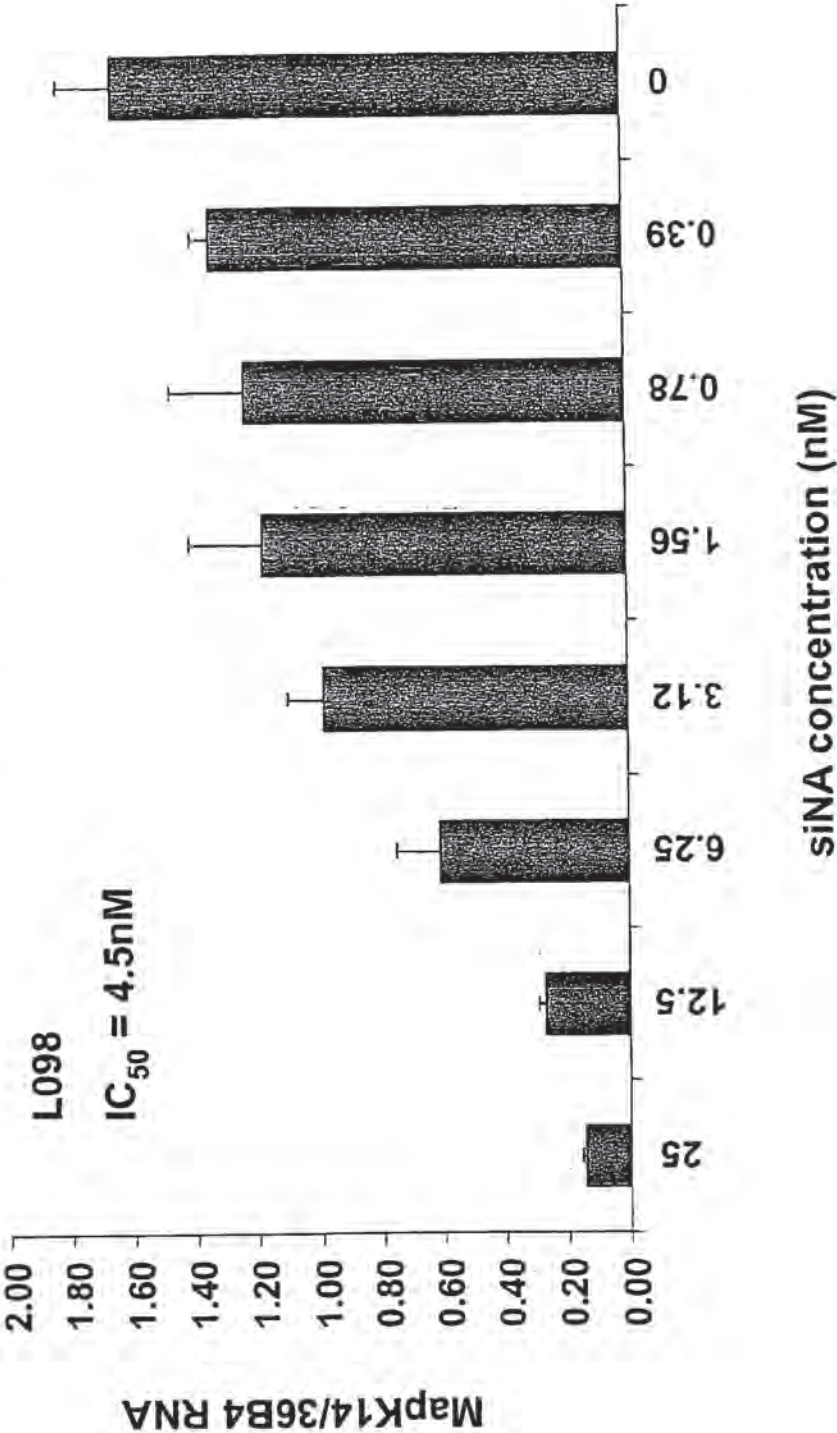
Figure 41
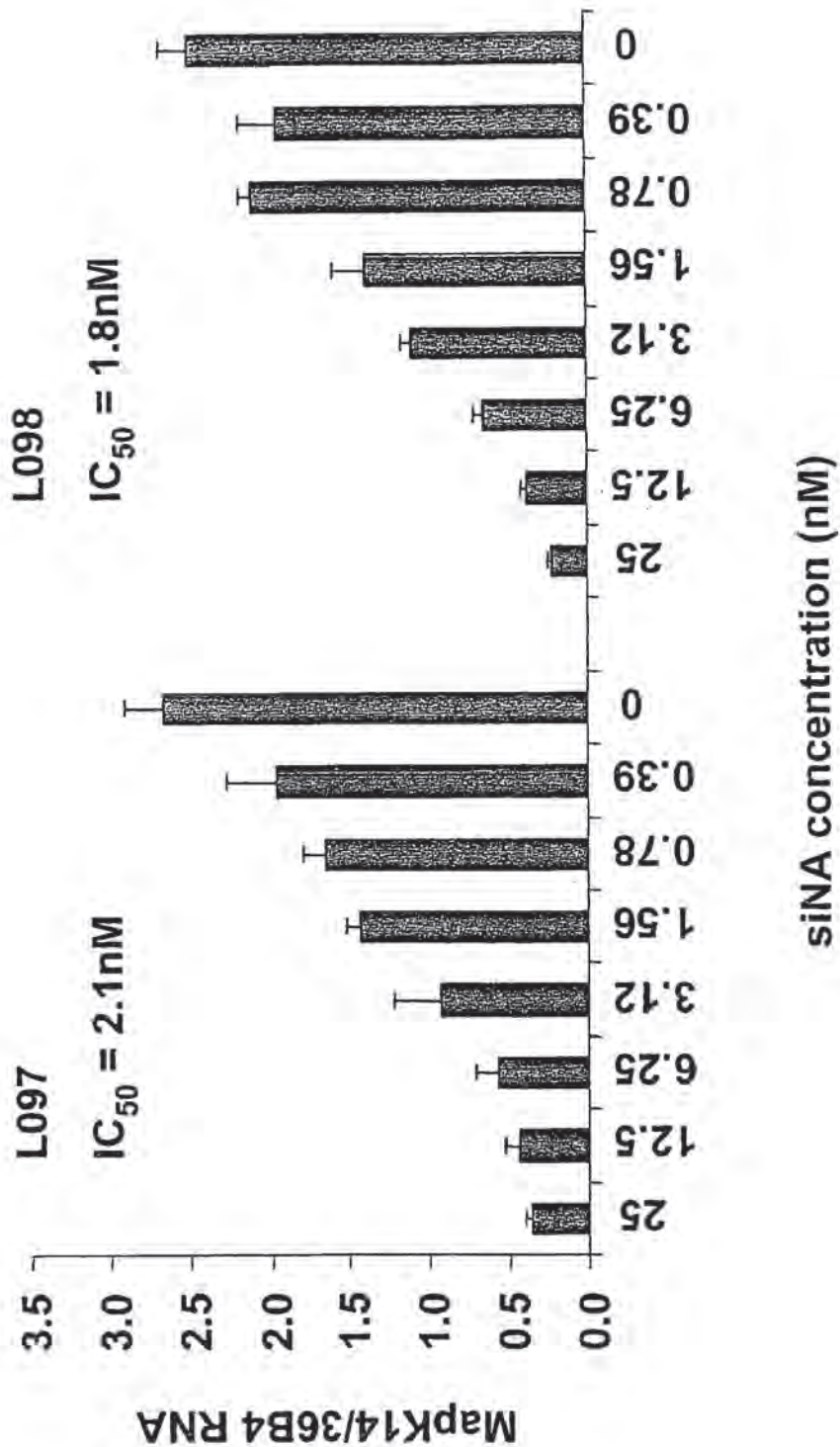
Figure 42
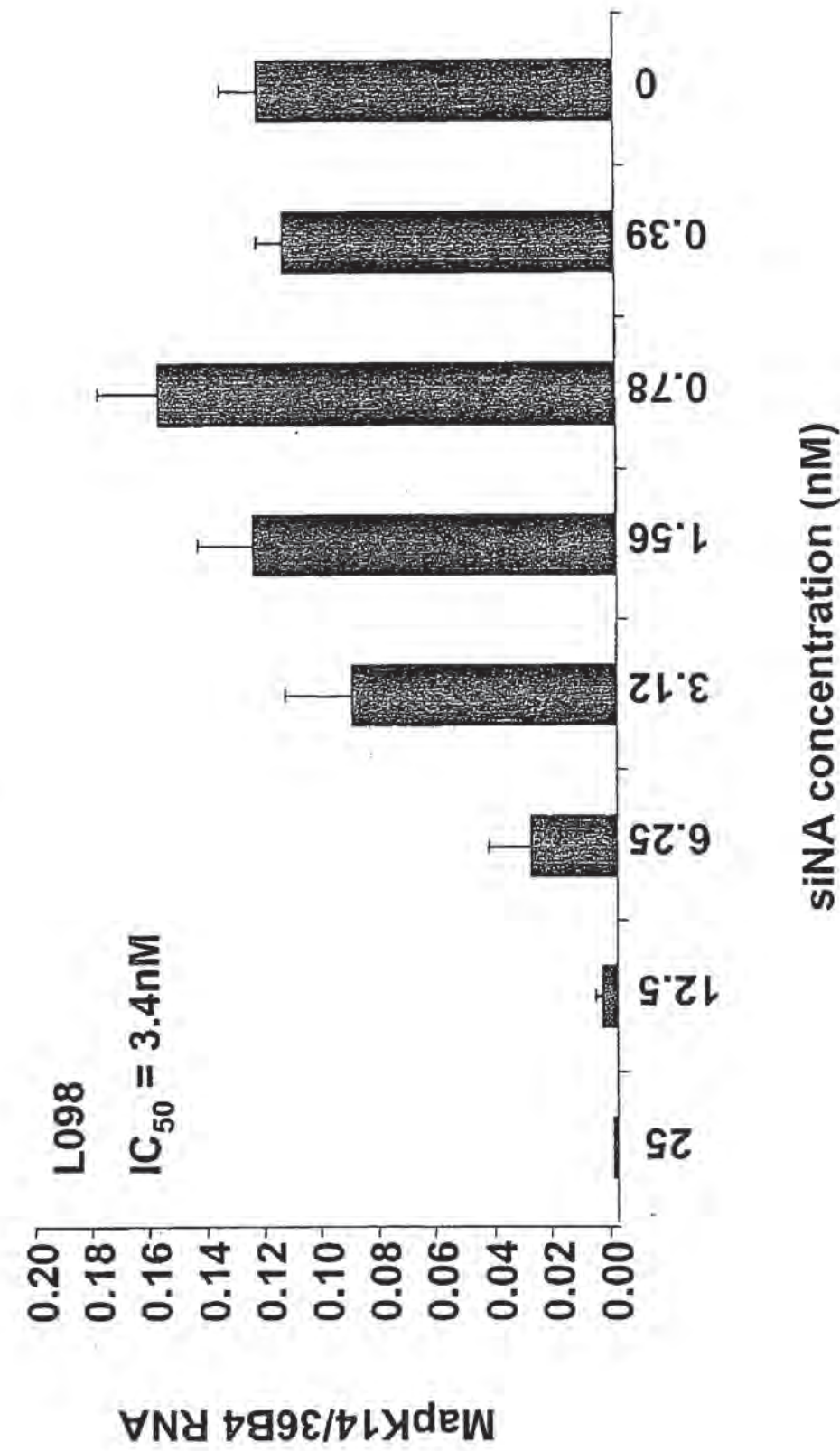
Figure 43
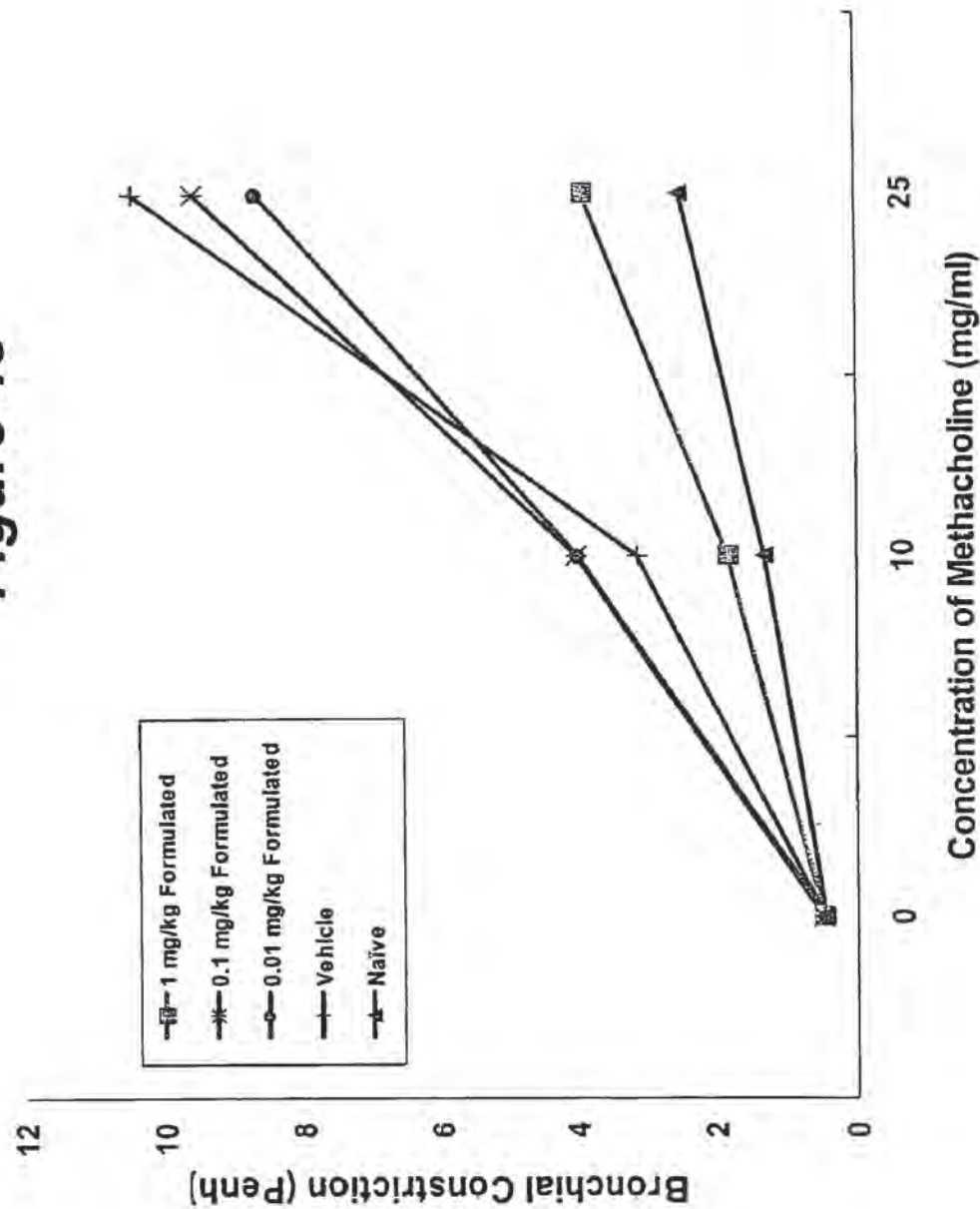
Figure 44: Relative expression of HD RNA with active LNP-98 and LNP-61 formulated active siNA compared to LNP-61 mismatch control

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LIPID NANOPARTICLE BASED COMPOSITIONS AND METHODS FOR THE DELIVERY OF BIOLOGICALLY ACTIVE MOLECULES

[0001] This application claims the benefit of U.S. Provisional patent application No. 60/652,787, filed Feb. 14, 2005, U.S. Provisional patent application No. 60/678,531, filed May 6, 2005, U.S. Provisional patent application No. 60/703,946, filed Jul. 29, 2005, and U.S. Provisional patent application No. 60/737,024, filed Nov. 15, 2005. These applications are incorporated by reference herein in their entirety including the drawings.

FIELD OF THE INVENTION

[0002] The present invention relates to novel particle forming delivery agents including cationic lipids, microparticles, and nanoparticles that are useful for delivering various molecules to cells. The invention also features compositions, and methods of use for the study, diagnosis, and treatment of traits, diseases and conditions that respond to the modulation of gene expression and/or activity in a subject or organism. Specifically, the invention relates to novel cationic lipids, microparticles, nanoparticles and transfection agents that effectively transfect or deliver biologically active molecules, such as antibodies (e.g., monoclonal, chimeric, humanized etc.), cholesterol, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, allozymes, aptamers, decoys and analogs thereof, and small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules, to relevant cells and/or tissues, such as in a subject or organism. Such novel cationic lipids, microparticles, nanoparticles and transfection agents are useful, for example, in providing compositions to prevent, inhibit, or treat diseases, conditions, or traits in a cell, subject or organism.

BACKGROUND OF THE INVENTION

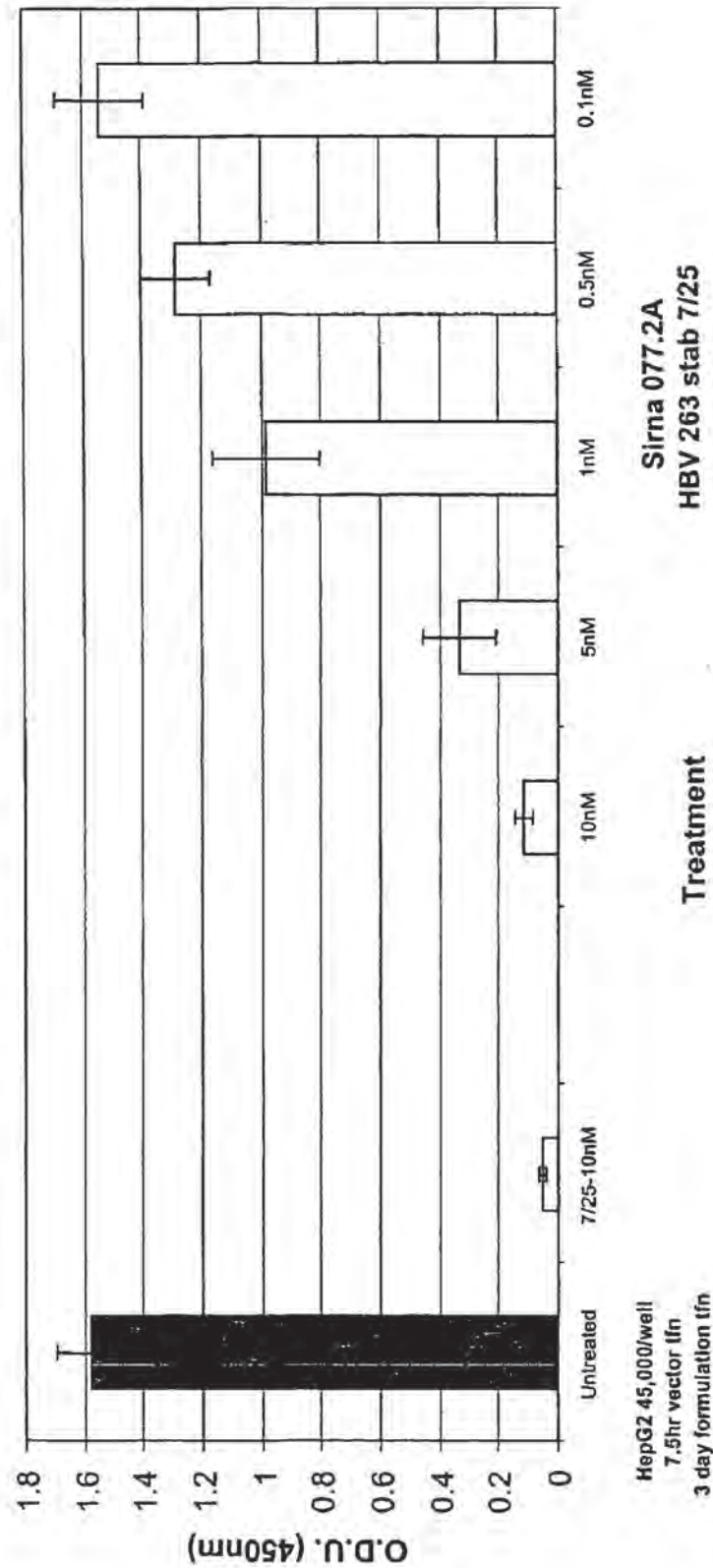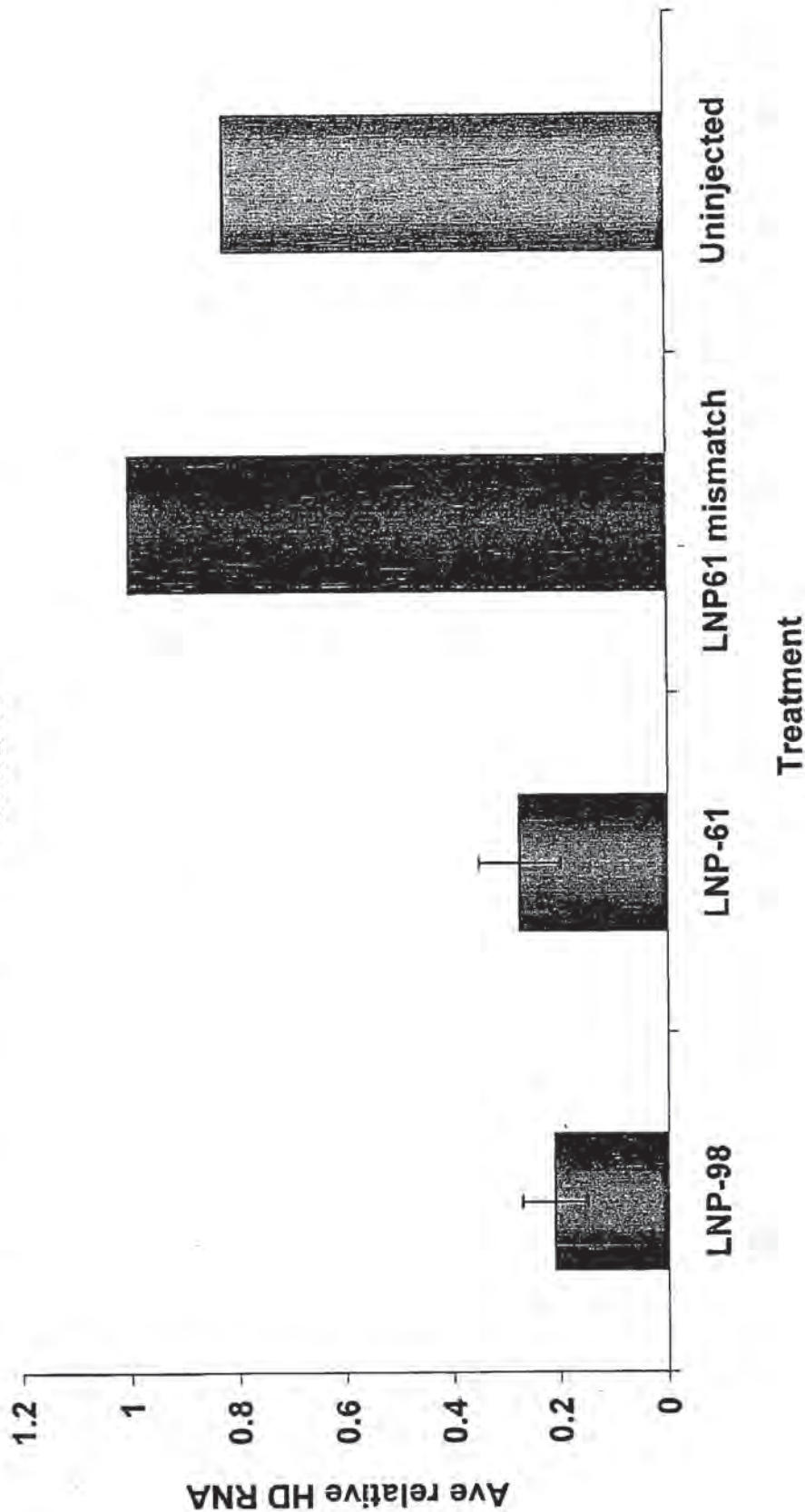[0003] The present invention relates to the delivery of biologically active molecules to cells. Specifically, the invention relates to compounds, compositions and methods for delivering nucleic acids, polynucleotides, and oligonucleotides such RNA, DNA and analogs thereof, peptides, polypeptides, proteins, antibodies, hormones and small molecules, to cells by facilitating transport across cellular membranes in, for example, epithelial tissues and endothelial tissues. The compounds, compositions and methods of the invention are useful in therapeutic, research, and diagnostic applications that rely upon the efficient transfer of biologically active molecules into cells, tissues, and organs. The discussion is provided only for understanding of the invention that follows. This summary is not an admission that any of the work described below is prior art to the claimed invention.

[0004] The cellular delivery of various therapeutic compounds, such as antiviral and chemotherapeutic agents, is usually compromised by two limitations. First the selectivity of a number of therapeutic agents is often low, resulting in high toxicity to normal tissues. Secondly, the trafficking of many compounds into living cells is highly restricted by the complex membrane systems of the cell. Specific transporters allow the selective entry of nutrients or regulatory molecules, while excluding most exogenous molecules such as nucleic acids and proteins. Various strategies can be used to improve transport of compounds into cells, including the use of lipid carriers, biodegradable polymers, and various conjugate systems.

[0005] The most well studied approaches for improving the transport of foreign nucleic acids into cells involve the use of viral vectors or cationic lipids and related cytofectins. Viral vectors can be used to transfer genes efficiently into some cell types, but they generally cannot be used to introduce chemically synthesized molecules into cells. An alternative approach is to use delivery formulations incorporating cationic lipids, which interact with nucleic acids through one end and lipids or membrane systems through another (for a review see Felgner, 1990, *Advanced Drug Delivery Reviews*, 5,162-187; Felgner 1993, *J. Liposome Res.*, 3,3-16). Synthetic nucleic acids as well as plasmids can be delivered using the cytofectins, although the utility of such compounds is often limited by cell-type specificity, requirement for low serum during transfection, and toxicity.

[0006] Another approach to delivering biologically active molecules involves the use of conjugates. Conjugates are often selected based on the ability of certain molecules to be selectively transported into specific cells, for example via receptor-mediated endocytosis. By attaching a compound of interest to molecules that are actively transported across the cellular membranes, the effective transfer of that compound into cells or specific cellular organelles can be realized. Alternately, molecules that are able to penetrate cellular membranes without active transport mechanisms, for example, various lipophilic molecules, can be used to deliver compounds of interest. Examples of molecules that can be utilized as conjugates include but are not limited to peptides, hormones, fatty acids, vitamins, flavonoids, sugars, reporter molecules, reporter enzymes, chelators, porphyrins, intercalcators, and other molecules that are capable of penetrating cellular membranes, either by active transport or passive transport.

[0007] The delivery of compounds to specific cell types, for example, cancer cells or cells specific to particular tissues and organs, can be accomplished by utilizing receptors associated with specific cell types. Particular receptors are overexpressed in certain cancerous cells, including the high affinity folic acid receptor. For example, the high affinity folate receptor is a tumor marker that is overexpressed in a variety of neoplastic tissues, including breast, ovarian, cervical, colorectal, renal, and nasoparyngeal tumors, but is expressed to a very limited extent in normal tissues. The use of folic acid based conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to the treatment and diagnosis of disease and can provide a reduction in the required dose of therapeutic compounds. Furthermore, therapeutic bioavailability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of bioconjugates, including folate bioconjugates. Godwin et al., 1972, *J. Biol. Chem.*, 247, 2266-2271, report the synthesis of biologically active pteroyloligo-L-glutamates. Habus et al., 1998, *Bioconjugate Chem.*, 9, 283-291, describe a method for the solid phase synthesis of certain oligonucleotide-folate con-

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jugates. Cook, U.S. Pat. No. 6,721,208, describes certain oligonucleotides modified with specific conjugate groups. The use of biotin and folate conjugates to enhance transmembrane transport of exogenous molecules, including specific oligonucleotides has been reported by Low et al., U.S. Pat. Nos. 5,416,016, 5,108,921, and International PCT publication No. WO 90/12096. Manoharan et al, International PCT publication No. WO 99/66063 describe certain folate conjugates, including specific nucleic acid folate conjugates with a phosphoramidite moiety attached to the nucleic acid component of the conjugate, and methods for the synthesis of these folate conjugates. Nomura et al., 2000, *J. Org. Chem.*, 65, 5016-5021, describe the synthesis of an intermediate, alpha-[2-(trimethylsilyl)ethoxycarbonyl]folic acid, useful in the synthesis of ceratin types of folate-nucleoside conjugates. Guzaev et al., U.S. Pat. No. 6,335,434, describes the synthesis of certain folate oligonucleotide conjugates. Vargeese et al., International PCT Publication No. WO 02/094185 and U.S. Patent Application Publication Nos. 20030130186 and 20040110296 describe certain nucleic acid conjugates.

[0008] The delivery of compounds to other cell types can be accomplished by utilizing receptors associated with a certain type of cell, such as hepatocytes. For example, drug delivery systems utilizing receptor-mediated endocytosis have been employed to achieve drug targeting as well as drug-uptake enhancement. The asialoglycoprotein receptor (ASGPr) (see for example Wu and Wu, 1987, *J. Biol. Chem.* 262, 4429-4432) is unique to hepatocytes and binds branched galactose-terminal glycoproteins, such as asialoorosomucoid (ASOR). Binding of such glycoproteins or synthetic glycoconjugates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain, for example, triatennary structures are bound with greater affinity than biatenarry or monoatennary chains (Baenziger and Fiete, 1980, *Cell*, 22, 611-620; Connolly et al., 1982, *J. Biol. Chem.*, 257, 939-945). Lee and Lee, 1987, *Glycoconjugate J.*, 4, 317-328, obtained this high specificity through the use of N-acetyl-D-galactosamine as the carbohydrate moiety, which has higher affinity for the receptor, compared to galactose. This "clustering effect" has also been described for the binding and uptake of mannosyl-terminating glycoproteins or glycoconjugates (Ponpipom et al., 1981, *J. Med. Chem.*, 24, 1388-1395). The use of galactose and galactosamine based conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to the treatment of liver disease such as HBV and HCV infection or hepatocellular carcinoma. The use of bioconjugates can also provide a reduction in the required dose of therapeutic compounds required for treatment. Furthermore, therapeutic bioavailability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of bioconjugates.

[0009] A number of peptide based cellular transporters have been developed by several research groups. These peptides are capable of crossing cellular membranes in vitro and in vivo with high efficiency. Examples of such fusogenic peptides include a 16-amino acid fragment of the homeodomain of ANTENNAPEDIA, a *Drosophila* transcription factor (Wang et al., 1995, *PNAS USA.*, 92, 3318-3322); a 17-mer fragment representing the hydrophobic region of the signal sequence of Kaposi fibroblast growth factor with or without NLS domain (Antopolsky et al., 1999, *Bioconj.*

Chem., 10, 598-606); a 17-mer signal peptide sequence of caiman crocodylus Ig(5) light chain (Chaloin et al., 1997, *Biochem. Biophys. Res. Comm.*, 243, 601-608); a 17-amino acid fusion sequence of HIV envelope glycoprotein gp4114, (Morris et al., 1997, *Nucleic Acids Res.*, 25, 2730-2736); the HIV-1 Tat49-57 fragment (Schwarze et al., 1999, *Science*, 285, 1569-1572); a transportan A—achimeric 27-mer consisting of N-terminal fragment of neuropeptide galanine and membrane interacting wasp venom peptide mastoporan (Lindgren et al., 2000, *Bioconjugate Chem.*, 11, 619-626); and a 24-mer derived from influenza virus hemagglutinin envelop glycoprotein (Bongartz et al., 1994, *Nucleic Acids Res.*, 22, 4681-4688). These peptides were successfully used as part of an antisense oligodeoxyribonucleotide-peptide conjugate for cell culture transfection without lipids. In a number of cases, such conjugates demonstrated better cell culture efficacy then parent oligonucleotides transfected using lipid delivery. In addition, use of phage display techniques has identified several organ targeting and tumor targeting peptides in vivo (Ruoslahti, 1996, *Ann. Rev. Cell Dev. Biol.*, 12, 697-715). Conjugation of tumor targeting peptides to doxorubicin has been shown to significantly improve the toxicity profile and has demonstrated enhanced efficacy of doxorubicin in the in vivo murine cancer model MDA-MB-435 breast carcinoma (Arap et al., 1998, *Science*, 279, 377-380).

[0010] Another approach to the intracellular delivery of biologically active molecules involves the use of cationic polymers. For example, Ryser et al., International PCT Publication No. WO 79/00515 describes the use of high molecular weight lysine polymers for increasing the transport of various molecules across cellular membranes. Rothbard et al., International PCT Publication No. WO 98/52614, describes certain methods and compositions for transporting drugs and macromolecules across biological membranes in which the drug or macromolecule is covalently attached to a transport polymer consisting of from 6 to 25 subunits, at least 50% of which contain a guanidino or amidino side chain. The transport polymers are preferably polyarginine peptides composed of all D-, all L- or mixtures of D- and L-arginine. Rothbard et al., U.S. Pat. No. Application Publication No. 20030082356, describes certain poly-lysine and poly-arginine compounds for the delivery of drugs and other agents across epithelial tissues, including the skin, gastrointestinal tract, pulmonary epithelium and blood brain barrier. Wendel et al., U.S. Patent Application Publication No. 20030032593, describes certain polyarginine compounds. Rothbard et al., U.S. Patent Application Publication No. 20030022831, describes certain poly-lysine and poly-arginine compounds for intra-ocular delivery of drugs. Kosak, U.S. Patent Application Publication No. 20010034333, describes certain cyclodextran polymers compositions that include a cross-linked cationic polymer component. Beigelman et al., U.S. Pat. No. 6,395,713; Reynolds et al., International PCT Publication No. WO 99/04819; Beigelman et al, International PCT Publication No. WO 99/05094; and Beigelman et al., U.S. Patent Application Publication No. 20030073640 describe certain lipid based formulations.

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[0011] Another approach to the intracellular delivery of biologically active molecules involves the use of liposomes or other particle forming compositions. Since the first description of liposomes in 1965, by Bangham (*J. Mol. Biol.* 13, 238-252), there has been a sustained interest and effort in the area of developing lipid-based carrier systems for the delivery of pharmaceutically active compounds. Liposomes are attractive drug carriers since they protect biological molecules from degradation while improving their cellular uptake. One of the most commonly used classes of liposome formulations for delivering polyanions (e.g., DNA) is that which contains cationic lipids. Lipid aggregates can be formed with macromolecules using cationic lipids alone or including other lipids and amphiphiles such as phosphatidylethanolamine. It is well known in the art that both the composition of the lipid formulation as well as its method of preparation have effect on the structure and size of the resultant anionic macromolecule-cationic lipid aggregate. These factors can be modulated to optimize delivery of polyanions to specific cell types in vitro and in vivo. The use of cationic lipids for cellular delivery of biologically active molecules has several advantages. The encapsulation of anionic compounds using cationic lipids is essentially quantitative due to electrostatic interaction. In addition, it is believed that the cationic lipids interact with the negatively charged cell membranes initiating cellular membrane transport (Akhtar et al., 1992, *Trends Cell Bio.*, 2, 139; Xu et al., 1996, *Biochemistry* 35, 5616).

[0012] Experiments have shown that plasmid DNA can be encapsulated in small particles that consist of a single plasmid encapsulated within a bilayer lipid vesicle (Wheeler, et al., 1999, Gene Therapy 6, 271-281). These particles typically contain the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE), low levels of a cationic lipid, and can be stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. These particles have systemic applications as they exhibit extended circulation lifetimes following intravenous (i.v.) injection, can accumulate preferentially in various tissues and organs or tumors due to the enhanced vascular permeability in such regions, and can be designed to escape the lyosomic pathway of endocytosis by disruption of endosomal membranes. These properties can be useful in delivering biologically active molecules to various cell types for experimental and therapeutic applications. For example, the effective use of nucleic acid technologies such as short interfering RNA (siRNA), antisense, ribozymes, decoys, triplex forming oligonucleotides, 2-5A oligonucleotides, and aptamers in vitro and in vivo may benefit from efficient delivery of these compounds across cellular membranes. Lewis et al., U.S. Patent Application Publication No. 20030125281, describes certain compositions consisting of the combination of siRNA, certain amphipathic compounds, and certain polycations. MacLachlan, U.S. Patent Application Publication No. 20030077829, describes certain lipid based formulations. MacLachlan, International PCT Publication No. WO 05/007196, describes certain lipid encapsulated interfering RNA formulations. Vargeese et al., International PCT Publication No. WO2005007854 describes certain polycationic compositions for the cellular delivery of polynucleotides. McSwiggen et al., International PCT Publication Nos. WO 05/019453, WO 03/70918, WO 03/74654 and U.S. Patent Application Publication Nos. 20050020525 and 20050032733, describes short interfering nucleic acid molecules (siNA) and various technologies for the delivery of siNA molecules and other polynucleotides.

[0013] In addition, recent work involving cationic lipid particles demonstrated the formation of two structurally different complexes comprising nucleic acid (or other polyanionic compound) and cationic lipid (Safinya et al, Science, 281: 78-81 (1998). One structure comprises a multilamellar structure with nucleic acid monolayers sandwiched between cationic lipid bilayers ("lamellar structure") (**FIG. 7**). A second structure comprises a two dimensional hexagonal columnar phase structure ("inverted hexagonal structure") in which nucleic acid molecules are encircled by cationic lipid in the formation of a hexagonal structure (**FIG. 7**). Safinya et al. demonstrated that the inverted hexagonal structure transfects mammalian cells more efficiently than the lamellar structure. Further, optical microscopy studies showed that the complexes comprising the lamellar structure bind stably to anionic vesicles without fusing to the vesicles, whereas the complexes comprising the inverted hexagonal structure are unstable and rapidly fuse to the anionic vesicles, releasing the nucleic acid upon fusion.

[0014] The structural transformation from lamellar phase to inverted hexagonal phase complexes is achieved either by incorporating a suitable helper lipid that assists in the adoption of an inverted hexagonal structure or by using a co-surfactant, such as hexanol. However, neither of these transformation conditions are suitable for delivery in biological systems. Furthermore, while the inverted hexagonal complex exhibits greater transfection efficiency, it has very poor serum stability compared to the lamellar complex. Thus, there remains a need to design delivery agents that are serum stable, i.e. stable in circulation, that can undergo structural transformation, for example from lamellar phase to inverse hexagonal phase, under biological conditions.

[0015] The present application provides compounds, compositions and methods for significantly improving the efficiency of systemic and local delivery of biologically active molecules. Among other things, the present application provides compounds, compositions and methods for making and using novel delivery agents that are stable in circulation and undergo structural changes under appropriate physiological conditions (e.g., pH) which increase the efficiency of delivery of biologically active molecules.

SUMMARY OF THE INVENTION

[0016] The present invention features compounds, compositions, and methods to facilitate delivery of various molecules into a biological system, such as cells. The compounds, compositions, and methods provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes or across one or more layers of epithelial or endothelial tissue. The present invention encompasses the design and synthesis of novel agents for the delivery of molecules, including but not limited to small molecules, lipids, nucleosides, nucleotides, nucleic acids, polynucleotides, oligonucleotides, antibodies, toxins, negatively charged polymers and other polymers, for example proteins, peptides, hormones, carbohydrates, or polyamines, across cellular membranes. Non-limiting examples of polynucleotides that can be delivered across cellular membranes using the compounds and methods of the invention include short interfering nucleic acids (siNA) (which includes siRNAs), antisense oligonucleotides, enzymatic nucleic acid molecules, 2',5'-oligoadenylates, triplex forming oligonucleotides, aptamers, and

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decoys. In general, the transporters described are designed to be used either individually or as part of a multi-component system, with or without degradable linkers. The compounds of the invention (generally shown in the Formulae below), when formulated into compositions, are expected to improve delivery of molecules into a number of cell types originating from different tissues, in the presence or absence of serum.

[0017] The compounds, compositions, and methods of the invention are useful for delivering biologically active molecules (e.g., siNAs, siRNAs, nucleic acids, polynucleotides, oligonucleotides, peptides, polypeptides, proteins, hormones, antibodies, and small molecules) to cells or across epithelial and endothelial tissues, such as skin, mucous membranes, vasculature tissues, gastrointestinal tissues, blood brain barrier tissues, opthamological tissues, pulmonary tissues, liver tissues, cardiac tissues, kidney tissues etc. The compounds, compositions, and methods of the invention can be used both for delivery to a particular site of administration or for systemic delivery.

[0018] The compounds, compositions, and methods of the invention can increase delivery or availability of biologically active molecules (e.g., siNAs, siRNAs, nucleic acids, polynucleotides, oligonucleotides, peptides, polypeptides, proteins, hormones, antibodies, and small molecules) to cells or tissues compared to delivery of the molecules in the absence of the compounds, compositions, and methods of the invention. As such, the level of a biologically active molecule inside a cell, tissue, or organism is increased in the presence of the compounds and compositions of the invention compared to when the compounds and compositions of the invention are absent.

[0019] In one aspect, the invention features novel cationic lipids, transfection agents, microparticles, nanoparticles, and formulations thereof with biologically active molecules. In another embodiment, the invention features compositions, and methods of use for the study, diagnosis, and treatment of traits, diseases, and conditions that respond to the modulation of gene expression and/or activity in a subject or organism. In another embodiment, the invention features novel cationic lipids, microparticles, nanoparticles transfection agents, and formulations that effectively transfect or deliver small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules, to relevant cells and/or tissues, such as in a subject or organism. Such novel cationic lipids, microparticles, nanoparticles, transfection agents, and formulations are useful, for example, in providing compositions to prevent, inhibit, or treat diseases, conditions, or traits in a cell, subject or organism as described herein.

[0020] In one aspect, the instant invention features various cationic lipids, microparticles, nanoparticles, transfection agents, and formulations for the delivery of chemically-modified synthetic short interfering nucleic acid (siNA) molecules that modulate target gene expression or activity in cells, tissues, such as in a subject or organism, by RNA interference (RNAi). The use of chemically-modified siNA improves various properties of native siRNA molecules through increased resistance to nuclease degradation in vivo, improved cellular uptake, and improved pharmacokinetic properties in vivo. The cationic lipids, microparticles, nanoparticles, transfection agents, formulations, and siNA molecules of the instant invention provide useful reagents and

methods for a variety of therapeutic, veterinary, diagnostic, target validation, genomic discovery, genetic engineering, and pharmacogenomic applications.

[0021] In one aspect, the invention features compositions and methods that independently or in combination modulate the expression of target genes encoding proteins, such as proteins associated with the maintenance and/or development of a disease, trait, or condition, such as a liver disease, trait, or condition. These genes are referred to herein generally as target genes. Such target genes are generally known in the art and transcripts of such genes are commonly referenced by Genbank Accession Number, see for example International PCT Publication No. WO 03/74654, serial No. PCT/US03/05028, and U.S. patent application Ser. No. 10/923,536 both incorporated by reference herein). The description below of the various aspects and embodiments of the invention is provided with reference to exemplary target genes and target gene transcripts. However, the various aspects and embodiments are also directed to other target genes, such as gene homologs, gene transcript variants, and gene polymorphisms (e.g., single nucleotide polymorphism, (SNPs)) that are associated with certain target genes. As such, the various aspects and embodiments are also directed to other genes that are involved in pathways of signal transduction or gene expression that are involved, for example, in the maintenance and/or development of a disease, trait, or condition. These additional genes can be analyzed for target sites using the methods described for target genes herein. Thus, the modulation of other genes and the effects of such modulation of the other genes can be performed, determined, and measured as described herein.

[0022] In one embodiment, the invention features a compound having Formula CLI:

CLI

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (for example, monoester, diester), or succinyl linker. In one embodiment, R1 and R2 are methyl, R3 is linoyl, L is butyl, and R4 is cholesterol, which compound is generally referred to herein as CLinDMA or 3-Dimethylamino-2-(Cholest-5-en-3β-oxybutan-4-oxy)-1-(cis,cis-9, 12-octadecadienoxy)propane.

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[0023]   In one embodiment, the invention features a compound having Formula CLII:

CLII

[0024]   wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester) or succinyl linker. In one embodiment, R1 and R2 are methyl, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0025]   In one embodiment, the invention features a compound having Formula CLIII:

CLIII
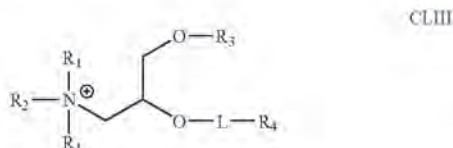
[0026]   wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In one embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, each R1 and R2 are methyl, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0027]   In one embodiment, the invention features a compound having Formula CLIV:

CLIV

[0028]   wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, each R1 and R2 are methyl, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0029]   In one embodiment, the invention features a compound having Formula CLV:

CLV

[0030]   wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; and each R3 and R4 is independently a C12-C24 aliphatic hydrocarbon, which can be the same or different. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each independently is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R1 and R2 are methyl, and R3 and R4 are oleyl, this compound is generally referred to herein as DMOBA or N,N-Dimethyl-3,4-dioleyloxybenzylamine.

[0031]   In one embodiment, the invention features a compound having Formula CLVI:

CLVI

[0032]   wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate,

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carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, R1 and R2 are methyl, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0033] In one embodiment, the invention features a compound having Formula CLVII:
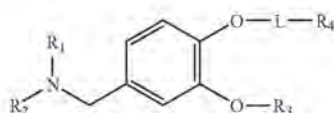
CLVII

[0034] wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon. L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, R1 and R2 are methyl, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0035] In one embodiment, the invention features a compound having Formula CLVIII:

CLVIII

[0036] wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; and each R3 and R4 is independently a C12-C24 aliphatic hydrocarbon which can be the same or different. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each independently is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, each R1 and R2 are methyl, and R3 and R4 are linoyl.

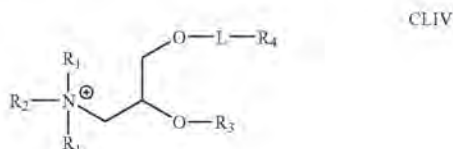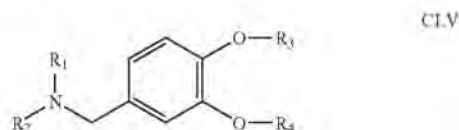[0037] In one embodiment, the invention features a compound having Formula CLIX:
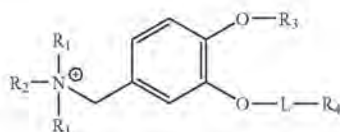
CLIX

[0038] wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24

aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamate carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, each R1 and R2 are methyl, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0039] In one embodiment, the invention features a compound having Formula CLX:
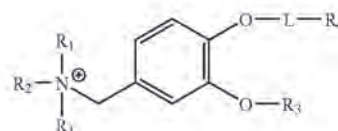
CLX

[0040] wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, each R1 and R2 are methyl, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0041] In one embodiment, the invention features a compound having Formula CLXI:
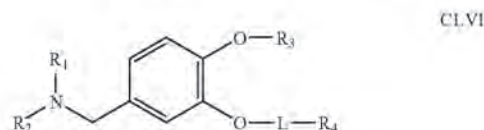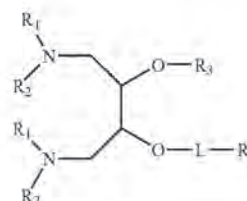

CLXI

[0042] wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodi-

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ment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, each R1 and R2 are methyl, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0043]   In one embodiment, the invention features a compound having Formula CLXIIa or CLXIIb:
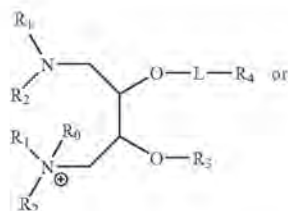
CLXIIa
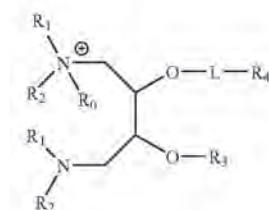
CLXIIb

[0044]   wherein R0 and each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, each R1 and R2 are methyl, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0045]   In one embodiment, the invention features a compound having Formula CLXIII:

CLXIII

[0046]   wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl,

petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, each R1 and R2 are methyl, R3 is linoyl, L is butyl, and R4 is cholesterol.
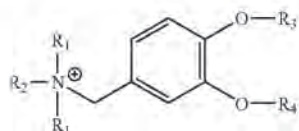
[0047]   In one embodiment, the invention features a compound having Formula CLXIVa and CLXIVb:

CLXIVa

CLXIVb

[0048]   wherein R0 and each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, each R1 and R2 are methyl, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0049]   In one embodiment, the invention features a compound having Formula CLXV:

CLXV

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[0050] wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; L is a linker, and each R3 is independently cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, each R1 and R2 are methyl, R3 is cholesterol, and L is butyl.

[0051] In one embodiment, the invention features a compound having Formula CLXVI:
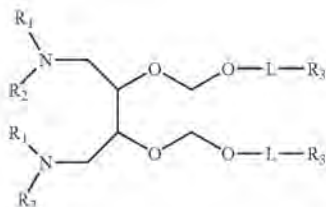
CLXVI

[0052] wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; each L is a linker whose structure is independent of the other L, and each R3 is independently cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, each R1 and R2 are methyl, R3 is cholesterol, and L is butyl.

[0053] In one embodiment, the invention features a compound having Formula CLXVII:

CLXVII

[0054] wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon and R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, each R1 and R2 are methyl and R3 is linoyl.

[0055] In one embodiment, the invention features a compound having Formula CLXVIII:

CLXVIII

[0056] wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, each R1 and R2 are methyl and R3 is linoyl.

[0057] In one embodiment, the invention features a compound having Formula CLXIX:

CLXIX

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 and R4 are each individually a C9-C24 aliphatic saturated or unsaturated hydrocarbon, which can be the same or different. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid.

[0058] In one embodiment, the invention features a compound having Formula CLXX:

CLXX

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 and R4 are each individually a C9-C24 aliphatic saturated or unsaturated hydrocarbon, which can be the same or different. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl, petroselinyl,

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linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid.

[0059]   In one embodiment, the invention features a compound having Formula CLXXI:
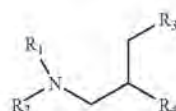
CLXXI

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 and R4 are each individually a C9-C24 aliphatic saturated or unsaturated hydrocarbon, which can be the same or different. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid.

[0060]   In one embodiment, the invention features a compound having Formula CLXXII:
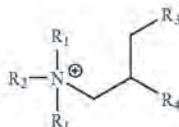
CLXXII

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 and R4 are each individually a C9-C24 aliphatic saturated or unsaturated hydrocarbon, which can be the same or different. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid.

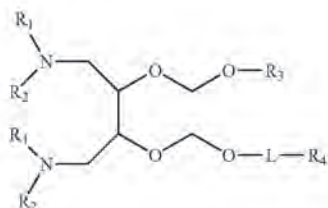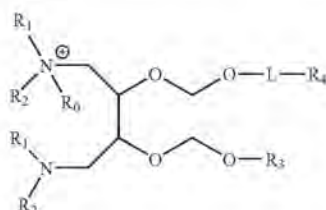[0061]   In one embodiment, the invention features a compound having Formula CLXXIII:

CLXXIII
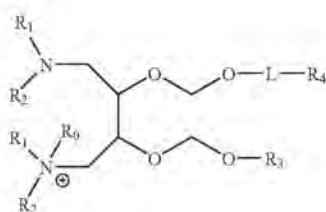
wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, and L is a linker. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl,

petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker.

[0062]   In one embodiment, the invention features a compound having Formula CLXXIV:
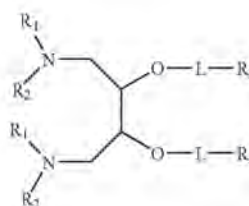

CLXXIV

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, and L is a linker. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker.

[0063]   In one embodiment, the invention features a compound having Formula CLXXV:

CLXXV

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, and L is a linker. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker.

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[0064] In one embodiment, the invention features a compound having Formula CLXXVI:

CLXXVI

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 and R4 are each individually a C9-C24 aliphatic saturated or unsaturated hydrocarbon, which can be the same or different. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid.
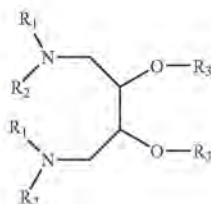
[0065] In one embodiment, the invention features a compound having Formula CLXXVII:

CLXXVII

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 and R4 are each individually a C9-C24 aliphatic saturated or unsaturated hydrocarbon, and L is a linker. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker.
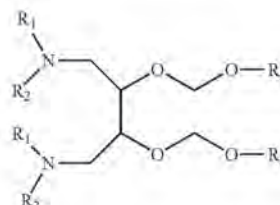
[0066] In one embodiment, the invention features a compound having Formula CLXXVIII:
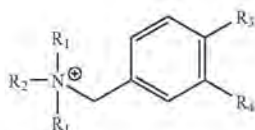
CLXXVIII

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 and R4 are each individually a C9-C24 aliphatic saturated or unsaturated hydrocarbon, which can be the same or different. In one embodiment,

R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid.
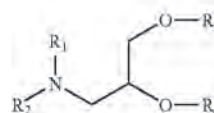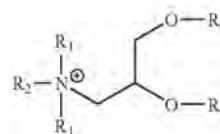
[0067] In one embodiment, the invention features a compound having Formula CLXXIX:
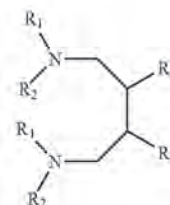
CLXXIX

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 and R4 are each individually a C9-C24 aliphatic saturated or unsaturated hydrocarbon, which can be the same or different. In one embodiment, R1 and R2 each independently is methyl, ethyl, propyl, isopropyl, or butyl. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid.

[0068] In one embodiment, the invention features a compound having Formula NLI:
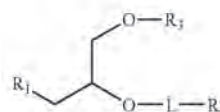
NLI

wherein R1 is H, OH, or a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon or alcohol; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 is OH, methyl, ethyl, propyl, isopropyl, or butyl or its corresponding alcohol. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (for example, monoester, diester), or succinyl linker. In one embodiment, R1 is OH, R3 is linoyl, L is butyl, and R4 is cholesterol.

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[0069] In one embodiment, the invention features a compound having Formula NLII:
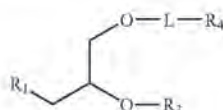
NLII

[0070] wherein R1 is H, OH, or a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon or alcohol; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 is methyl, ethyl, propyl, isopropyl, or butyl or its corresponding alcohol. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester) or succinyl linker. In one embodiment, R1 is OH, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0071] In one embodiment, the invention features a compound having Formula NLIII:
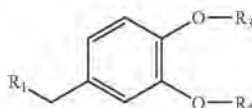
NLIII

[0072] wherein R1 is H, OH, a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon or alcohol; and each R3 and R4 is independently a C12-C24 aliphatic hydrocarbon, which can be the same or different. In one embodiment, R1 is methyl, ethyl, propyl, isopropyl, or butyl or its corresponding alcohol. In one embodiment, R3 and R4 each independently is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R1 is OH, and R3 and R4 are oleyl, this compound is generally referred to herein as DOBA or dioleyloxybenzyl alcohol.

[0073] In one embodiment, the invention features a compound having Formula NLIV:
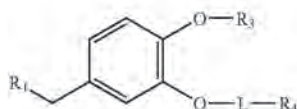
NLIV

[0074] wherein R1 is H, OH a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon or alcohol; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 is methyl, ethyl, propyl,

isopropyl, or butyl or its corresponding alcohol. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, R1 is OH, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0075] In one embodiment, the invention features a compound having Formula NLV:
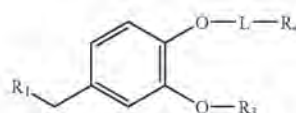
NLV

[0076] wherein R1 is H, OH a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon or alcohol; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L is a linker, and R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid. In one embodiment, R1 is methyl, ethyl, propyl, isopropyl, or butyl or its corresponding alcohol. In one embodiment, R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R4 is cholesterol. In one embodiment, L is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, L is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker. In one embodiment, R1 is OH, R3 is linoyl, L is butyl, and R4 is cholesterol.

[0077] In one embodiment, the invention features a compound having Formula NLVI:
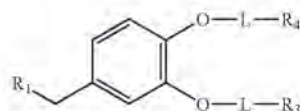
NLVI

wherein R1 is H, OH, a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon or alcohol; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, and each L is a linker. In one embodiment, R1 is methyl, ethyl, propyl, isopropyl, or butyl or its corresponding alcohol. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholestero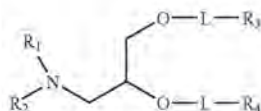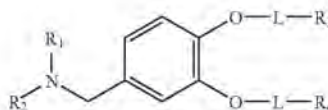l derivative, a steroid hormone, or a bile acid. In one embodiment, each L independently is a C1 to C10 alkyl, alkyl ether, polyether, or polyethylene glycol linker. In another embodiment, each L independently is an acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl linker.

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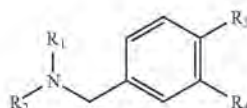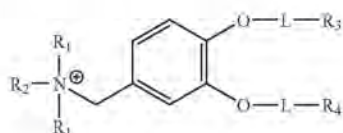
Oct. 26, 2006

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[0078]   In one embodiment, the invention features a compound having Formula NLVII:
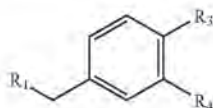
NLVII

wherein R1 is independently H, OH, a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon or alcohol; R3 and R4 are each individually a C9-C24 aliphatic saturated or unsaturated hydrocarbon, which can be the same or different. In one embodiment, R1 is methyl, ethyl, propyl, isopropyl, or butyl or its corresponding alcohol. In one embodiment, R3 and R4 each individually is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl. In one embodiment, R3 or R4 is cholesterol, a cholesterol derivative, a steroid hormone, or a bile acid.

[0079]   In one embodiment, each O—R3 and/or O—R4 of any compound having Formulae CLI-CLXIV, CLXVII-CLXXII, CLXXVI, and CLXXVIII-CLXXIX further comprises a linker L (e.g., wherein —O—R3 and/or —O—R4 as shown above is —O-L-R3 and/or —O-L-R4), where L is a C1 to C10 alkyl, alkyl ether, polyether, polyethylene glycol, acetal, amide, succinyl, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or other linker as is generally known in the art.

[0080]   In one embodiment, a formulation of the invention (e.g., a formulated molecular compositions (FMC) or lipid nanoparticle (LNP) of the invention) is a neutral lipid having any of formulae NLI-NLVII.

[0081]   Examples of a steroid hormone include those comprising cholesterol, estrogen, testosterone, progesterone, glucocortisone, adrenaline, insulin, glucagon, cortisol, vitamin D, thyroid hormone, retinoic acid, and/or growth hormones.

[0082]   In one embodiment, the invention features a composition comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, other nucleic acid molecule or other biologically active molecule described herein), a cationic lipid, a neutral lipid, and a polyethyleneglycol conjugate, such as a PEG-diacylglycerol, PEG-diacylglycamide, PEG-cholesterol, or PEG-DMB conjugate. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative. The compositions described herein are generally referred to as formulated molecular compositions (FMC) or lipid nanoparticles (LNP). In some embodiments of the invention, a formulated molecular composition (FMC) or lipid nanoparticle (LNP) composition further comprises cholesterol or a cholesterol derivative.

[0083]   Suitable cationic lipid include those cationic lipids which carry a net negative charge at a selected pH, such as physiological pH. Particularly useful cationic lipids include those having a relatively small head group, such as a tertiary amine, quaternary amine or guanidine head group, and sterically hindered asymmetric lipid chains. In any of the

embodiments described herein, the cationic lipid can be selected from those comprising Formulae CLI, CLII, CLIII, CLIV, CLV, CLVI, CLVII, CLVIII, CLIX, CLX, CLXI, CLXII, CLXIII, CLXIV, CLXV, CLXVI, CLXVI, CLXVII, CLXVIII, CLXIX, CLXX, CLXXI, CLXXII, CLXXIII, CLXXIV, CLXXV, CLXXVI, CLXXVII, CLXXVIII, CLXXIX: N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(1-(2,3-dioleylox-y)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2,3-dioleyloxy)propylamine (DODMA), 1,2-Dioleoyl-3-Dimethylammonium-propane (DODAP), 1,2-Dioleoylcarbamyl-3-Dimethylammonium-propane (DOCDAP), 1,2-Dilineoyl-3-Dimethylammonium-propane (DLINDAP), Dioleoyloxy-N-[2-sperminecarboxamido)et-hyl}-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), Dioctadecylamidoglycyl spermine (DOGS), DC-Chol, 1,2-Dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE), 3-Dimethylamino-2-(Cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octa-decadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3β-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',12'-octadecadienoxy)propane (CpLinDMA), N,N-Dimethyl-3, 4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-Dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), and/or a mixture thereof, as well as other cationic lipids sharing similar properties. The above cationic lipids can include various differing salts as are known in the art. Non-limiting examples of these cationic lipid structures are shown in **FIGS. 1-5** and **FIG. 19**.

[0084]   In some embodiments, the head group of the cationic lipid can be attached to the lipid chain via a cleavable or non-cleavable linker, such as a linker described herein or otherwise known in the art. Non-limiting examples of suitable linkers include those comprising a C1 to C10 alkyl, alkyl ether, polyether, polyethylene glycol, acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester (i.e., monoester, diester), or succinyl.

[0085]   Suitable neutral lipids include those comprising any of a variety of neutral uncharged, zwitterionic or anionic lipids capable of producing a stable complex. They are preferably neutral, although they can alternatively be positively or negatively charged. In any of the embodiments described herein, suitable neutral lipids include those selected from compounds having formulae NLI-NLVII, dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), -phosphatidylethanolamine (POPE) and dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), cholesterol, as well as other neutral lipids described herein below, and/or a mixture thereof.

[0086]   Suitable polyethyleneglycol-diacylglycerol or polyethyleneglycol-diacylglycamide (PEG-DAG) conjugates include those comprising a dialkylglycerol or dialkylglycamide group having alkyl chain length independently comprising from about C4 to about C40 saturated or unsaturated carbon atoms. The dialkylglycerol or dialkylglycamide group can further comprise one or more substituted alkyl

groups. In any of the embodiments described herein, the PEG conjugate can be selected from PEG-dilaurylglycerol (C12), PEG-dimyristylglycerol (C14), PEG-dipalmitoylg-lycerol (C16), PEG-disterylglycerol (C18), PEG-dilaurylg-lycamide (C12), PEG-dimyristylglycamide (C14), PEG-di-palmitoylglycamide (C16), and PEG-disterylglycamide (C18), PEG-cholesterol (1-[8'-(Cholest-5-en-3β-oxy)car-boxamido-3',6'-dioxaoctanyl]carbamoyl-ω-methyl-poly-(ethylene glycol), and PEG-DMB (3,4-Ditetradecoxylben-zyl-ω-methyl-poly(ethylene glycol)ether).

[0087] In one embodiment, the invention features a com-position comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule) fomulated as L051, L053, L054, L060, L061, L069, L073, L077, L080, L082, L083, L086, L097, L098, L099, L100, L101, L102, L103, and/or L104 (see Table IV).

[0088] Other suitable PEG conjugates include PEG-cho-lesterol or PEG-DMB conjugates (see for example **FIG. 24**). In one embodiment, PEG conjugates include PEGs attached to saturated or unsaturated lipid chains such as oleyl, linoleyl and similar lipid chains.

[0089] In one embodiment, the invention features a com-position comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), a cationic lipid having any of Formulae CLI-CLXXIX, a neutral lipid, and a PEG-DAG (i.e., polyethyleneglycol-diacylglycerol or polyethylenegly-col-diacylglycamide), PEG-cholesterol, or PEG-DMB con-jugate. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative. In another embodiment, the composition is formulated as L051, L053, L054, L060, L061, L069, L073, L077, L080, L082, L083, L086, L097, L098, L099, L100, L101, L102, L103, and/or L104 herein (see Table IV).

[0090] In one embodiment, the invention features a com-position comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), a cationic lipid comprising 3-Dim-ethylamino-2-(Cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis, cis-9,12-octadecadienoxy)propane (CLinDMA), a neutral lipid comprising distearoylphosphatidylcholine (DSPC), a PEG-DAG comprising PEG-n-dimyristylglycerol (PEG-DMG), and cholesterol. In one embodiment, the molar ratio of CLinDMA:DSPC:cholesterol:PEG-DMG are 48:40:10:2 respectively, this composition is generally referred to herein as formulation L051.

[0091] In one embodiment, the invention features a com-position comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), a cationic lipid comprising N,N-Dimethyl-3,4-dioleyloxybenzylamine (DMOBA), a neutral lipid comprising distearoylphosphatidylcholine (DSPC), a PEG-DAG comprising PEG-n-dimyristylglycerol (PEG-DMG), and cholesterol. In one embodiment, the molar ratio of DMOBA:DSPC:cholesterol:PEG-DMG are 30:20:48:2 respectively, this composition is generally referred to herein as formulation L053.

[0092] In one embodiment, the invention features a com-position comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), a cationic lipid comprising N,N-Dimethyl-3,4-dioleyloxybenzylamine (DMOBA), a neutral lipid comprising distearoylphosphatidylcholine (DSPC), a PEG-DAG comprising PEG-n-dimyristylglycerol (PEG-DMG), and cholesterol. In one embodiment, the molar ratio of DMOBA:DSPC:cholesterol:PEG-DMG are 50:20:28:2 respectively, this composition is generally referred to herein as formulation L054. In another embodiment, the composi-tion further comprises a neutral lipid, such as dioleoylphos-phatidylethanolamine (DOPE), palmitoyloleoylphosphati-dylcholine (POPC), egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC), cholesterol, and/or a mixture thereof.

[0093] In one embodiment, the invention features a com-position comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), a cationic lipid comprising compris-ing 3-Dimethylamino-2-(Cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), a cationic lipid comprising N,N-Dimethyl-3,4-dioleyloxy-benzylamine (DMOBA), a neutral lipid comprising dis-tearoylphosphatidylcholine (DSPC), a PEG-DAG compris-ing PEG-n-dimyristylglycerol (PEG-DMG), and cholesterol. In one embodiment, the molar ratio of CLinD-MA:DMOBA:DSPC:cholesterol:PEG-DMG are 25:25:20:28:2 respectively, this composition is generally referred to herein as formulation L073.

[0094] In one embodiment, the invention features a com-position comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), a cationic lipid comprising compris-ing 3-Dimethylamino-2-(Cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), a neutral lipid comprising distearoylphosphatidylcholine (DSPC), a PEG comprising PEG-Cholesterol (PEG-Chol), and cholesterol. In one embodiment, the molar ratio of CLinDMA:DSPC:cholesterol:PEG-Chol are 48:40:10:2 respectively, this composition is generally referred to herein as formulation L069.

[0095] In one embodiment, the invention features a com-position comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), a cationic lipid comprising compris-ing 1,2-N,N'-Dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), a neutral lipid comprising distearoylphos-phatidylcholine (DSPC), a PEG-DAG comprising PEG-n-dimyristylglycerol (PEG-DMG), and cholesterol. In one embodiment, the molar ratio of DOcarbDAP:DSPC:choles-terol:PEG-DMG are 30:20:48:2 respectively, this composi-tion is generally referred to herein as formulation T018.1.

[0096] In one embodiment, the invention features a com-position comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), a cationic lipid comprising compris-

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ing N,N-dimethyl-2,3-dioleyloxy)propylamine (DODMA), a neutral lipid comprising distearoylphosphatidylcholine (DSPC), a PEG-DAG comprising PEG-n-dimyristylglycerol (PEG-DMG), and cholesterol. In one embodiment, the molar ratio of DODMA:DSPC:cholesterol:PEG-DMG are 30:20:48:2 respectively, this composition is generally referred to herein as formulation T019.1.

[0097] In one embodiment, the invention features a composition comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), and a cationic lipid comprising a compound having any of Formula CLI, CLII, CLIII, CLIV, CLV, CLVI, CLVII, CLVIII, CLIX, CLX, CLXI, CLXII, CLXIII, CLXIV, CLXV, CLXVI, CLXVII, CLXVIII, CLXIX, CLXX, CLXXI, CLXXII, CLXXIII, CLXXIV, CLXXV, CLXXVI, CLXXVII, CLXXVIII, CLXXIX. In another embodiment, the composition further comprises a neutral lipid, such as dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC), cholesterol, and/or a mixture thereof. In another embodiment, the composition further comprises a PEG conjugate. In yet another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0098] In one embodiment, the invention features a composition comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), and a cationic lipid comprising 3-Dimethylamino-2-(Cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA). In another embodiment, the composition further comprises a neutral lipid, such as dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC), cholesterol, and/or a mixture thereof. In another embodiment, the composition further comprises a PEG conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB). In yet another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0099] In one embodiment, the invention features a composition comprising a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), and a cationic lipid comprising N,N-Dimethyl-3,4-dioleyloxybenzylamine (DMOBA). In another embodiment, the composition further comprises a neutral lipid, such as dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC), cholesterol, and/or a mixture thereof. In yet another embodiment, the composition further comprises the cationic lipid CLinDMA. In another embodiment, the composition further comprises a PEG conjugate. In yet another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0100] The term "biologically active molecule" as used herein refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system. Non-limiting examples of biologically active molecules

include antibodies (e.g., monoclonal, chimeric, humanized etc.), cholesterol, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2.5-A chimeras, siNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of modulating the pharmacokinetics and/or pharmacodynamics of other biologically active molecules, for example, lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers. In certain embodiments, the term biologically active molecule is used interchangeably with the term "molecule" or "molecule of interest" herein.

[0101] In one embodiment, the invention features a composition comprising a siNA molecule, a cationic lipid having any of Formulae CLI-CLXXIX, a neutral lipid, and a polyethyleneglycol-diacylglycerol or polyethyleneglycol-diacylglycamide (PEG-DAG) conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB). These compositions are generally referred to herein as formulated siNA compositions. In another embodiment, a formulated siNA composition of the invention further comprises cholesterol or a cholesterol derivative.

[0102] In one embodiment, the siNA component of a formulated siNA composition of the invention is chemically modified so as not to stimulate an interferon response in a mammalian cell, subject, or organism. Such siNA molecules can be said to have improved toxicologic profiles, such as having attenuated or no immunostimulatory properties, having attenuated or no off-target effect, or otherwise as described herein.

[0103] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol-diacylglycerol (PEG-DAG) conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against RNA of a target gene, wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to the target gene RNA for the siNA molecule to mediate RNA interference against the target gene RNA. In one embodiment, the target RNA comprises RNA sequence referred to by Genbank Accession numbers in International PCT Publication No. WO 03/74654, serial No. PCT/US03/05028, and U.S. patent application Ser. No. 10/923,536 both incorporated by reference herein. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0104] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol-diacylglycerol (PEG-DAG) conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against a Hepatitis Virus RNA, wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises

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nucleotide sequence having sufficient complementarity to the Hepatitis Virus RNA for the siNA molecule to mediate RNA interference against the Hepatitis Virus RNA. In one embodiment, the Hepatitis Virus RNA is Hepatitis B Virus (HBV). In one embodiment, the Hepatitis Virus RNA is Hepatitis C Virus (HCV). In one embodiment, the siNA comprises sequences described in U.S. Patent Application Nos. 60/401,104, Ser. Nos. 10/667,271, and 10/942,560, which are incorporated by reference in their entireties herein. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0105] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against Protein Tyrosine Phosphatase 1B (PTP1B) RNA, wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to the PTP1B RNA for the siNA molecule to mediate RNA interference against the PTP1B RNA. In one embodiment, the siNA comprises sequences described in U.S. Patent Application Publication Nos. 20040019001 and 200500704978, which are incorporated by reference in their entireties herein. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0106] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against Transforming Growth Factor beta (TGF-beta) and/or Transforming Growth Factor beta Receptor (TGF-betaR) RNA, wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to the TGF-beta and/or TGF-betaR RNA for the siNA molecule to mediate RNA interference against the TGF-beta and/or TGF-betaR RNA. In one embodiment, the siNA comprises sequences described in U.S. Ser. No. 11/054,047, which is incorporated by reference in their entireties herein. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0107] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against cholesteryl ester transfer protein (CETP) RNA, wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to the CETP RNA for the siNA molecule to mediate RNA interference against the CETP RNA. In one embodiment, the siNA comprises sequences described in U.S. Ser. No. 10/921,554, which is incorporated by reference in its entirety herein. In

another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0108] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against Gastric Inhibitory Peptide (GIP) RNA, wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to the GIP RNA for the siNA molecule to mediate RNA interference against the GIP RNA. In one embodiment, the siNA comprises sequences described in U.S. Ser. No. 10/916,030, which is incorporated by reference in its entirety herein. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0109] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against Stearoyl-CoA Desaturase (SCD) RNA, wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to the SCD RNA for the siNA molecule to mediate RNA interference against the SCD RNA. In one embodiment, the siNA comprises sequences described in U.S. Ser. No. 10/923,451, which is incorporated by reference in its entirety herein. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0110] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol-diacylglycerol conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against Acetyl-CoA carboxylase (ACACB) RNA, wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to the ACACB RNA for the siNA molecule to mediate RNA interference against the ACACB RNA. In one embodiment, the siNA comprises sequences described in U.S. Ser. No. 10/888,226, which is incorporated by reference in its entirety herein. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0111] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against apolipoprotein RNA (e.g., apo AI, apo A-IV, apo B, apo C-III, and/or apo E

RNA), wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to the apolipoprotein RNA for the siNA molecule to mediate RNA interference against the apolipoprotein RNA. In one embodiment, the siNA comprises sequences described in U.S. Ser. No. 11/054,047, which is incorporated by reference in their entireties herein. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0112] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against VEGF and/or VEGF-receptor RNA (e.g., VEGF, VEGFR1, VEGFR2 and/or VEGFR3 RNA), wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to the VEGF and/or VEGF-receptor RNA for the siNA molecule to mediate RNA interference against the VEGF and/or VEGF-receptor RNA. In one embodiment, the siNA comprises sequences described in U.S. Ser. No. 10/962,898, which is incorporated by reference in their entireties herein. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0113] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG_DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against IL4-receptor RNA, wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to the IL4-receptor RNA for the siNA molecule to mediate RNA interference against the IL4-receptor RNA. In one embodiment, the siNA comprises sequences described in U.S. Ser. No. 11/001,347, which is incorporated by reference in their entireties herein. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0114] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG_DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against Hairless RNA, wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to the Hairless RNA for the siNA molecule to mediate RNA interference against the Hairless RNA. In one embodiment, the siNA comprises sequences described in U.S. Ser. No. 10/919,964, which is incorporated by reference in their entireties herein. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0115] In one embodiment, the invention features a composition comprising: (a) a cationic lipid having any of Formulae CLI-CLXXIX; (b) a neutral lipid; (c) a polyethyleneglycol conjugate (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG_DMB); and (d) a short interfering nucleic acid (siNA) molecule that mediates RNA interference (RNAi) against a target RNA, wherein each strand of said siNA molecule is about 18 to about 28 nucleotides in length; and one strand of said siNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the siNA molecule to mediate RNA interference against the target RNA. In one embodiment, the target RNA comprises RNA sequence referred to by Genbank Accession numbers in International PCT Publication No. WO 03/74654, serial No. PCT/US03/05028, and U.S. patent application Ser. No. 10/923,536 both incorporated by reference herein. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative.

[0116] In one embodiment, the cationic lipid component (e.g., a compound having any of Formulae CLI-CLXXIX or as otherwise described herein) of a composition of invention comprises from about 2% to about 60%, from about 5% to about 45%, from about 5% to about 15%, or from about 40% to about 50% of the total lipid present in the formulation.

[0117] In one embodiment, the neutral lipid component of a composition of the invention comprises from about 5% to about 90%, or from about 20% to about 85% of the total lipid present in the formulation.

[0118] In one embodiment, the PEG conjugate (i.e., PEG_DAG, PEG-cholesterol, PEG-DMB) of a composition of the invention comprises from about 1% to about 20%, or from about 4% to about 15% of the total lipid present in the formulation.

[0119] In one embodiment, the cholesterol component of a composition of the invention comprises from about 10% to about 60%, or from about 20% to about 45% of the total lipid present in the formulation.

[0120] In one embodiment, a formulated siNA composition of the invention comprises a cationic lipid component comprising from about 30 to about 50% of the total lipid present in the formulation, a neutral lipid comprising from about 30 to about 50% of the total lipid present in the formulation, and a PEG conjugate (i.e., PEG_DAG, PEG-cholesterol, PEG-DMB) comprising about 0 to about 10% of the total lipid present in the formulation.

[0121] In one embodiment, a formulated molecular composition of the invention comprises a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), a compound having any of Formulae CLI-CLXXIX, DSPC, and a PEG conjugate (i.e., PEG-DAG, PEG-cholesterol, PEG-DMB). In one embodiment, the PEG conjugate is PEG-dilaurylglycerol (C12), PEG-dimyristylglycerol (C14), PEG-dipalmitoylglycerol (C16), or PEG-disterylglycerol (C18). In another embodiment, the PEG conjugate is PEG-dilaurylglycamide (C12), PEG-dimyristylglycamide (C14), PEG-dipalmitoylglycamide (C16), or PEG-disterylglycamide (C18). In another embodiment, the PEG conjugate is PEG-cholesterol or PEG-DMB. In another embodiment, the formulated molecular composition further comprises cholesterol or a cholesterol derivative.

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[0122] In one embodiment, a formulated molecular composition of the invention comprises a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), a compound having Formula CLI, DSPC, and a PEG conjugate. In one embodiment, the PEG conjugate is PEG-dilaurylglycerol (C12), PEG-dimyristylglycerol (C14), PEG-dipalmitoylglycerol (C16), or PEG-disterylglycerol (C18). In another embodiment, the PEG conjugate is PEG-dilaurylglycamide (C12), PEG-dimyristylglycamide (C14), PEG-dipalmitoylglycamide (C16), or PEG-disterylglycamide (C18). In another embodiment, the PEG conjugate is PEG-cholesterol or PEG-DMB. In another embodiment, the formulated molecular composition further comprises cholesterol or a cholesterol derivative.

[0123] In one embodiment, a formulated molecular composition of the invention comprises a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule), a compound having Formula CLV, DSPC, and a PEG conjugate. In one embodiment, the PEG conjugate is PEG-dilaurylglycerol (C12), PEG-dimyristylglycerol (C14), PEG-dipalmitoylglycerol (C16), or PEG-disterylglycerol (C18). In another embodiment, the PEG conjugate is PEG-dilaurylglycamide (C12), PEG-dimyristylglycamide (C14), PEG-dipalmitoylglycamide (C16), or PEG-disterylglycamide (C18). In another embodiment, the PEG conjugate is PEG-cholesterol or PEG-DMB. In another embodiment, the formulated molecular composition further comprises cholesterol or a cholesterol derivative.

[0124] In one embodiment, a composition of the invention (e.g., a formulated molecular composition) further comprises a targeting ligand for a specific cell of tissue type. Non-limiting examples of such ligands include sugars and carbohydrates such as galactose, galactosamine, and N-acetyl galactosamine; hormones such as estrogen, testosterone, progesterone, glucocortisone, adrenaline, insulin, glucagon, cortisol, vitamin D, thyroid hormone, retinoic acid, and growth hormones; growth factors such as VEGF, EGF, NGF, and PDGF; cholesterol; bile acids; neurotransmitters such as GABA, Glutamate, acetylcholine; NOGO; inostitol triphosphate; diacylglycerol; epinephrine; norepinephrine; Nitric Oxide, peptides, vitamins such as folate and pyridoxine, drugs, antibodies and any other molecule that can interact with a receptor in vivo or in vitro. The ligand can be attached to any component of a formulated siNA composition of invention (e.g., cationic lipid component, neutral lipid component, PEG-DAG component, or siNA component etc.) using a linker molecule, such as an amide, amido, carbonyl, ester, peptide, disulphide, silane, nucleoside, abasic nucleoside, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, phosphate ester, phosphoramidate, thiophosphate, alkylphosphate, or photolabile linker. In one embodiment, the linker is a biodegradable linker.

[0125] In one embodiment, the PEG conjugate of the invention, such as a PEG-DAG, PEG-cholesterol, PEG-DMB, comprises a 200 to 10,000 atom PEG molecule.

[0126] In one embodiment, the compositions of the present invention, e.g., a formulated molecular composition, comprise a diacylglycerol-polyethyleneglycol conjugate,

i.e., a DAG-PEG conjugate. The term "diacylglycerol" refers to a compound having 2-fatty acyl chains, R1 and R2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Diacylglycerols have the following general Formula VIII:
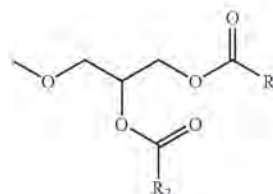
[0127] wherein R1 and R2 are each an alkyl, substituted alkyl, aryl, substituted aryl, lipid, or a ligand. In one embodiment, R1 and R2 are each independently a C2 to C30 alkyl group. In one embodiment, the DAG-PEG conjugate is a dilaurylglycerol (C 12)-PEG conjugate, a dimyristylglycerol (C14)-PEG conjugate, a dipalmitoylglycerol (C16)-PEG conjugate, a disterylglycerol (C18)-PEG conjugate, PEG-dilaurylglycamide (C12), PEG-dimyristylglycamide (C14), PEG-dipalmitoylglycamide (C16), or PEG-disterylglycamide (C18). Those of skill in the art will readily appreciate that other diacylglycerols can be used in the DAG-PEG conjugates of the present invention.

[0128] In one embodiment, the compositions of the present invention, e.g., a formulated molecular composition, comprise a polyethyleneglycol-cholesterol conjugate, i.e., a PEG-chol conjugate. The PEG-chol conjugate can comprise a 200 to 10,000 atom PEG molecule linked to cholesterol or a cholesterol derivative. An exemplary PEG-chol and the synthesis thereof is shown in FIG. 24.

[0129] In one embodiment, the compositions of the present invention, e.g., a formulated molecular composition, comprise a polyethyleneglycol-DMB conjugate. The term "DMB" refers to the compound 3,4-Ditetradecoxy]benzyl-β-methyl-poly(ethylene glycol) ether. The PEG-DMB conjugate can comprise a 200 to 10,000 atom PEG molecule linked to DMB. An exemplary PEG-DMB and the synthesis thereof is shown in FIG. 24.

[0130] The term "ligand" refers to any compound or molecule, such as a drug, peptide, hormone, or neurotransmitter that is capable of interacting with another compound, such as a receptor, either directly or indirectly. The receptor that interacts with a ligand can be present on the surface of a cell or can alternately be an intercellular receptor. Interaction of the ligand with the receptor can result in a biochemical reaction, or can simply be a physical interaction or association. Non-limiting examples of ligands include sugars and carbohydrates such as galactose, galactosamine, and N-acetyl galactosamine; hormones such as estrogen, testosterone, progesterone, glucocortisone, adrenaline, insulin, glucagon, cortisol, vitamin D, thyroid hormone, retinoic acid, and growth hormones; growth factors such as VEGF, EGF, NGF, and PDGF; cholesterol; bile acids; neurotransmitters such as GABA, Glutamate, acetylcholine; NOGO; inostitol triphosphate; diacylglycerol; epinephrine; norepi-

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nephrine; Nitric Oxide, peptides, vitamins such as folate and pyridoxine, drugs, antibodies and any other molecule that can interact with a receptor in vivo or in vitro. The ligand can be attached to a compound of the invention using a linker molecule, such as an amide, amido, carbonyl, ester, peptide, disulphide, silane, nucleoside, abasic nucleoside, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, phosphate ester, phosphoramidate, thiophosphate, alkylphosphate, or photolabile linker. In one embodiment, the linker is a biodegradable linker.

[0131] The term "degradable linker" as used herein, refers to linker moieties that are capable of cleavage under various conditions. Conditions suitable for cleavage can include but are not limited to pH, UV irradiation, enzymatic activity, temperature, hydrolysis, elimination, and substitution reactions, and thermodynamic properties of the linkage.

[0132] The term "photolabile linker" as used herein, refers to linker moieties as are known in the art that are selectively cleaved under particular UV wavelengths. Compounds of the invention containing photolabile linkers can be used to deliver compounds to a target cell or tissue of interest, and can be subsequently released in the presence of a UV source.

[0133] The term "lipid" as used herein, refers to any lipophilic compound. Non-limiting examples of lipid compounds include fatty acids and their derivatives, including straight chain, branched chain, saturated and unsaturated fatty acids, carotenoids, terpenes, bile acids, and steroids, including cholesterol and derivatives or analogs thereof.

[0134] In addition to the foregoing components, the compositions of the present invention can further comprise cationic poly(ethylene glycol) (PEG) lipids, or CPLs, that have been designed for insertion into lipid bilayers to impart a positive charge (see for example Chen, et al., 2000, Bioconj. Chem. 11, 433-437). Suitable formulations for use in the present invention, and methods of making and using such formulations are disclosed, for example in U.S. application Ser. No. 09/553,639, which was filed Apr. 20, 2000, and PCT Patent Application No. CA 00/00451, which was filed Apr. 20, 2000 and which published as WO 00/62813 on Oct. 26, 2000, the teachings of each of which is incorporated herein in its entirety by reference.

[0135] In one embodiment, the compositions of the present invention, i.e., those formulated molecular compositions containing PEG conjugates, are made using any of a number of different methods. In one embodiment, the present invention provides lipid-nucleic acid particles produced via hydrophobic polynucleotide-lipid intermediate complexes. The complexes are preferably charge-neutralized. Manipulation of these complexes in either detergent-based or organic solvent-based systems can lead to particle formation in which the nucleic acid is protected.

[0136] In one embodiment, the present invention provides a serum-stable formulated molecular composition (e.g., comprising a biologically active molecules such as polynucleotides including siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecules) in which the biologically active molecule is encapsulated in a lipid bilayer and is protected from degradation (for example, where the composition adopts a lamellar structure). Additionally, the formulated particles formed in the present invention are preferably

neutral or negatively-charged at physiological pH. In one embodiment, for in vivo applications, neutral particles can be advantageous, while for in vitro applications the particles can be negatively charged. This provides the further advantage of reduced aggregation over the positively-charged liposome formulations in which a biologically active molecule can be encapsulated in cationic lipids.

[0137] In addition, the present invention provides serum-stable formulated molecular compositions that undergo a rapid pH-dependent phase transition. The pH-dependent phase transition results in a structural change that increases the efficiency of delivery of a biologically active molecule, such as a polynucleotide, into a biological system, such as a cell. The structural change can increase the efficiency of delivery by, for example, increasing cell membrane fusion and release of a biologically active molecule into a biological system. Thus, in one embodiment, the serum-stable formulated molecular composition is stable in plasma or serum (i.e., in circulation) and stable at physiologic pH (i.e., about pH 7.4) and undergoes a rapid pH-dependent phase transition resulting in a structural change that increases the efficiency of delivery of a biologically active molecule into a biological system. In one embodiment, the pH dependent phase transition occurs at about pH 5.5-6.5. In one embodiment, the serum-stable formulated molecular composition undergoes a structural change to adopt an inverted hexagonal structure at about pH 5.5-6.5. For example, the serum-stable formulated molecular composition can transition from a stable lamellar structure adopted in circulation (i.e., in plasma or serum) at physiologic pH (about pH 7.4) to a less stable and more efficient delivery composition having an inverted hexagonal structure at pH 5.5-6.5, which is the pH found in the early endosome. The serum-stable formulated molecular compositions that undergo a rapid pH-dependent phase transition demonstrate increased efficiency in the delivery of biologically active molecules due to their stability in circulation at physiologic pH and their ability to undergo a pH dependent structural change that increases cell membrane fusion and release of a biologically active molecule into a biological system, such as a cell.

[0138] The serum-stable formulated molecular composition that undergoes a rapid pH-dependent phase transition comprises a biologically active molecule (e.g., a polynucleotide such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, other nucleic acid molecule or other biologically active molecule described herein), a cationic lipid, a neutral lipid, and a polyethylene conjugate such as a polyethyleneglycol-diacylglycerol, polyethyleneglycol-diacylglycamide, polyethyleneglycol-cholesterol or polyethylene-DMB conjugate. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative. Examples of suitable cationic lipids, neutral lipids, and PEG conjugates are provided herein.

[0139] In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is CLinDMA, the neutral lipid is distearoylphosphatidylcholine (DSPC), and the PEG conjugate is PEG-DMG. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative. This is known as formulation L051 (see Table IV).

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[0140]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is DMOBA, the neutral lipid is distearoylphosphatidylcholine (DSPC), and the PEG conjugate is PEG-DMG. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative. This is known as formulation L053 or L054 (see Table IV).

[0141]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is CLinDMA, the neutral lipid is distearoylphosphatidylcholine (DSPC), and the PEG conjugate is 2KPEG-cholesterol. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative. This is known as formulation L069 (see Table IV).

[0142]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is pCLinDMA or CLinDMA and DMOBA, the neutral lipid is distearoylphosphatidylcholine (DSPC), and the PEG conjugate is PEG-DMG. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative. This is known as formulation L073 (see Table IV).

[0143]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is eCLinDMA, the neutral lipid is distearoylphosphatidylcholine (DSPC), and the PEG conjugate is 2KPEG-cholesterol. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative. This is known as formulation L077 (see Table IV).

[0144]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is eCLinDMA, the neutral lipid is distearoylphosphatidylcholine (DSPC), and the PEG conjugate is 2KPEG-DMG. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative. This is known as formulation L080 (see Table IV).

[0145]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is pCLinDMA, the neutral lipid is distearoylphosphatidylcholine (DSPC), and the PEG conjugate is 2KPEG-DMG. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative. This is known as formulation L082 (see Table IV).

[0146]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is pCLinDMA, the neutral lipid is distearoylphosphatidylcholine (DSPC), and the PEG conjugate is 2KPEG-cholesterol. In another embodiment, the compo-

sition further comprises cholesterol or a cholesterol derivative. This is known as formulation L083 (see Table IV).

[0147]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is CLinDMA, the neutral lipid is distearoylphosphatidylcholine (DSPC), and the PEG conjugate is 2KPEG-DMG. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative and Linoleyl alcohol. This is known as formulation L086 (see Table IV).

[0148]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is DMLBA, the neutral lipid is cholesterol, and the PEG conjugate is 2KPEG-DMG. This is known as formulation L061 (see Table IV).

[0149]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is DMOBA, the neutral lipid is cholesterol, and the PEG conjugate is 2KPEG-DMG, and the nitrogen to phoshpate (N/P) ratio of the formulated molecular composition is 5. This is known as formulation L060 (see Table IV).

[0150]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is DMLBA, the neutral lipid is cholesterol, and the PEG conjugate is 2KPEG-DMG. This is known as formulation L097 (see Table IV).

[0151]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is DMOBA, the neutral lipid is cholesterol, and the PEG conjugate is 2KPEG-DMG, and the nitrogen to phoshpate (N/P) ratio of the formulated molecular composition is 3. This is known as formulation L098 (see Table IV).

[0152]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is DMOBA, the neutral lipid is cholesterol, and the PEG conjugate is 2KPEG-DMG, and the nitrogen to phoshpate (N/P) ratio of the formulated molecular composition is 4. This is known as formulation L099 (see Table IV).

[0153]   In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is DMOBA, the neutral lipid is DOBA, and the PEG conjugate is 2KPEG-DMG (3%), and the nitrogen to phoshpate (N/P) ratio of the formulated molecular composition is 3. This is known as formulation L100 (see Table IV).

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[0154] In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is DMOBA, the neutral lipid is cholesterol, and the PEG conjugate is 2K-PEG-Cholesterol. This is known as formulation L101 (see Table IV).

[0155] In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is DMOBA, the neutral lipid is cholesterol, and the PEG conjugate is 2K-PEG-Cholesterol, and the nitrogen to phoshpate (N/P) ratio of the formulated molecular composition is 5. This is known as formulation L102 (see Table IV).

[0156] In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is DMLBA, the neutral lipid is cholesterol, and the PEG conjugate is 2K-PEG-Cholesterol. This is known as formulation L103 (see Table IV).

[0157] In one embodiment, the invention features a serum-stable formulated molecular composition comprising a biologically active molecule (e.g., a siNA molecule), a cationic lipid, a neutral lipid, and a PEG-conjugate, in which the cationic lipid is CLinDMA, the neutral lipid is distearoylphosphatidylcholine (DSPC), and the PEG conjugate is 2KPEG-cholesterol. In another embodiment, the composition further comprises cholesterol or a cholesterol derivative and Linoleyl alcohol. This is known as formulation L104 (see Table IV).

[0158] The invention additionally provides methods for determining whether a formulated molecular composition will be effective for delivery of a biologically active molecule into a biological system. In one embodiment, the method for determining whether a formulated molecular composition will be effective for delivery of a biologically active molecule into a biological system comprises (1) measuring the serum stability of the formulated molecular composition and (2) measuring the pH dependent phase transition of the formulated molecular composition, wherein a determination that the formulated molecular composition is stable in serum and a determination that the formulated molecular composition undergoes a phase transition at about pH 4 to about 7, e.g., from 5.5 to 6.5, indicates that the formulated molecular composition will be effective for delivery of a biologically active molecule into a biological system. In another embodiment, the method further comprises measuring the transfection efficiency of the formulated molecular composition in a cell in vitro.

[0159] The serum stability of the formulated molecular composition can be measured using any assay that measures the stability of the formulated molecular composition in serum, including the assays described herein and otherwise known in the art. One exemplary assay that can be used to measure the serum stability is an assay that measures the relative turbidity of the composition in serum over time. For example, the relative turbidity of a formulated molecular composition can be determined by measuring the absorbance of the formulated molecular composition in the pres-

ence or absence of serum (i.e., 50%) at several time points over a 24 hour period using a spectrophotometer. The formulated molecular composition is stable in serum if the relative turbidity, as measured by absorbance, remains constant at around 1.0 over time.

[0160] The pH dependent phase transition of the formulated molecular composition can be measured using any assay that measures the phase transition of the formulated molecular composition at about pH 5.5-6.5, including the assays described herein and otherwise known in the art. One exemplary assay that can be used to measure the pH dependent phase transition is an assay that measures the relative turbidity of the composition at different pH over time. For example, the relative turbidity of a formulated molecular composition can be determined by measuring the absorbance over time of the formulated molecular composition in buffer having a range of different pH values. The formulated molecular composition undergoes pH dependent phase transition if the relative turbidity, as measured by absorbance, decreases when the pH drops below 7.0.

[0161] In addition, the efficiency of the serum-stable formulated molecular composition that undergoes a rapid pH-dependent phase transition as a delivery agent can be determined by measuring the transfection efficiency of the formulated molecular composition. Methods for performing transfection assays are described herein and otherwise known in the art.

[0162] In one embodiment, the particles made by the methods of this invention have a size of about 50 to about 600 nm. The particles can be formed by either a detergent dialysis method or by a modification of a reverse-phase method which utilizes organic solvents to provide a single phase during mixing of the components. Without intending to be bound by any particular mechanism of formation, a molecule (e.g., a biologically active molecule such as a polynucleotide) is contacted with a detergent solution of cationic lipids to form a coated molecular complex. These coated molecules can aggregate and precipitate. However, the presence of a detergent reduces this aggregation and allows the coated molecules to react with excess lipids (typically, noncationic lipids) to form particles in which the molecule of interest is encapsulated in a lipid bilayer. The methods described below for the formation of formulated molecular compositions using organic solvents follow, a similar scheme.

[0163] In one embodiment, the particles are formed using detergent dialysis. Thus, the present invention provides a method for the preparation of serum-stable formulated molecular compositions, including those that undergo pH dependent phase transition, comprising: (a) combining a molecule (e.g., a biologically active molecule such as a polynucleotide, including siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecules) with cationic lipids in a detergent solution to form a coated molecule-lipid complex; (b) contacting noncationic lipids with the coated molecule-lipid complex to form a detergent solution comprising a siNA-lipid complex and noncationic lipids; and (c) dialyzing the detergent solution of step (b) to provide a solution of serum-stable molecule-lipid particles, wherein the molecule is encapsulated in a lipid bilayer and the particles are serum-stable and have a size of from about 50 to about 600 nm.

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[0164] In one embodiment, an initial solution of coated molecule-lipid (e.g., polynucleotide-lipid) complexes is formed, for example, by combining the molecule with the cationic lipids in a detergent solution.

[0165] In these embodiments, the detergent solution is preferably an aqueous solution of a neutral detergent having a critical micelle concentration of 15-300 mM, more preferably 20-50 mM. Examples of suitable detergents include, for example, N,N'-((octanoylimino)-bis-(trimethylene))-bis-(D-gluconamide) (BIGCHAP); BRIJ 35; Deoxy-BIGC-HAP; dodecylpoly(ethylene glycol) ether; Tween 20; Tween 40; Tween 60; Tween 80; Tween 85; Mega 8; Mega 9; Zwittergent® 3-08; Zwittergent® 3-10; Triton X-405; hexyl-, heptyl-, octyl- and nonyl-beta-D-glucopyranoside; and heptylthioglucopyranoside; with octyl β-D-glucopyranoside and Tween-20 being the most preferred. The concentration of detergent in the detergent solution is typically about 100 mM to about 2 M, preferably from about 200 mM to about 1.5 M.

[0166] In one embodiment, the cationic lipids and the molecule of interest (e.g., a biologically active molecule such as a polynucleotide, including siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecules) will typically be combined to produce a charge ratio (+/−) of about 1:1 to about 20:1, preferably in a ratio of about 1:1 to about 12:1, and more preferably in a ratio of about 2:1 to about 6:1. Additionally, the overall concentration of siNA in solution will typically be from about 25 µg/mL to about 1 mg/mL, preferably from about 25 µg/mL to about 500 µg/mL, and more preferably from about 100 µg/mL to about 250 µg/mL. The combination of the molecules of interest and cationic lipids in detergent solution is kept, typically at room temperature, for a period of time which is sufficient for the coated complexes to form. Alternatively, the molecules of interest and cationic lipids can be combined in the detergent solution and warmed to temperatures of up to about 37° C. For molecules (e.g., certain polynucleotides herein) which are particularly sensitive to temperature, the coated complexes can be formed at lower temperatures, typically down to about 4° C.

[0167] In one embodiment, the siNA to lipid ratios (mass/mass ratios) in a formed formulated molecular composition range from about 0.01 to about 0.08. The ratio of the starting materials also falls within this range because the purification step typically removes the unencapsulated siNA as well as the empty liposomes. In another embodiment, the formulated siNA composition preparation uses about 400 µg siNA per 10 mg total lipid or a siNA to lipid ratio of about 0.01 to about 0.08 and, more preferably, about 0.04, which corresponds to 1.25 mg of total lipid per 50 µg of siNA. A formulated molecular composition of the invention is developed to target specific organs, tissues, or cell types. In one embodiment, a formulated molecular composition of the invention is developed to target the liver or hepatocytes. Ratios of the various components of the formulated molecular composition are adjusted to target specific organs, tissues, or cell types.

[0168] In one embodiment, the invention features a method for delivering or administering a biologically active molecule to a cell or cells in a subject or organism, comprising administering a formulated molecular composition

of the invention under conditions suitable for delivery of the biologically active molecule component of the formulated molecular composition to the cell or cells of the subject or organism. In one embodiment, the formulated molecular composition is contacted with the cell or cells of the subject or organism as is generally known in the art, such as via parental administration (e.g., intravenous, intramuscular, subcutaneous administration) of the formulated molecular composition with or without excipients to facilitate the administration.

[0169] In one embodiment, the invention features a method for delivering or administering a biologically active molecule to liver or liver cells (e.g., hepatocytes) in a subject or organism, comprising administering a formulated molecular composition of the invention under conditions suitable for delivery of the biologically active molecule component of the formulated molecular composition to the liver or liver cells (e.g., hepatocytes) of the subject or organism. In one embodiment, the formulated molecular composition is contacted with the liver or liver cells of the subject or organism as is generally known in the art, such as via parental administration (e.g., intravenous, intramuscular, subcutaneous administration) or local administration (e.g., direct injection, portal vein injection, catheterization, stenting etc.) of the formulated molecular composition with or without excipients to facilitate the administration.

[0170] In one embodiment, the invention features a method for delivering or administering a biologically active molecule to kidney or kidney cells in a subject or organism, comprising administering a formulated molecular composition of the invention under conditions suitable for delivery of the biologically active molecule component of the formulated molecular composition to the kidney or kidney cells of the subject or organism. In one embodiment, the formulated molecular composition is contacted with the kidney or kidney cells of the subject or organism as is generally known in the art, such as via parental administration (e.g., intravenous, intramuscular, subcutaneous administration) or local administration (e.g., direct injection, catheterization, stenting etc.) of the formulated molecular composition with or without excipients to facilitate the administration.

[0171] In one embodiment, the invention features a method for delivering or administering a biologically active molecule to tumor or tumor cells in a subject or organism, comprising administering a formulated molecular composition of the invention under conditions suitable for delivery of the biologically active molecule component of the formulated molecular composition to the tumor or tumor cells of the subject or organism. In one embodiment, the formulated molecular composition is contacted with the tumor or tumor cells of the subject or organism as is generally known in the art, such as via parental administration (e.g., intravenous, intramuscular, subcutaneous administration) or local administration (e.g., direct injection, catheterization, stenting etc.) of the formulated molecular composition with or without excipients to facilitate the administration.

[0172] In one embodiment, the invention features a method for delivering or administering a biologically active molecule to CNS or CNS cells (e.g., brain, spinal cord) in a subject or organism, comprising administering a formulated molecular composition of the invention under conditions suitable for delivery of the biologically active molecule

component of the formulated molecular composition to the CNS or CNS cells of the subject or organism. In one embodiment, the formulated molecular composition is contacted with the CNS or CNS cells of the subject or organism as is generally known in the art, such as via parental administration (e.g., intravenous, intramuscular, subcutaneous administration) or local administration (e.g., direct injection, catheterization, stenting etc.) of the formulated molecular composition with or without excipients to facilitate the administration.

[0173] In one embodiment, the invention features a method for delivering or administering a biologically active molecule to lung or lung cells in a subject or organism, comprising administering a formulated molecular composition of the invention under conditions suitable for delivery of the biologically active molecule component of the formulated molecular composition to the lung or lung cells of the subject or organism. In one embodiment, the formulated molecular composition is contacted with the lung or lung cells of the subject or organism as is generally known in the art, such as via parental administration (e.g., intravenous, intramuscular, subcutaneous administration) or local administration (e.g., pulmonary administration directly to lung tissues and cells) of the formulated molecular composition with or without excipients to facilitate the administration.

[0174] In one embodiment, the invention features a method for delivering or administering a biologically active molecule to vascular or vascular cells in a subject or organism, comprising administering a formulated molecular composition of the invention under conditions suitable for delivery of the biologically active molecule component of the formulated molecular composition to the vascular or vascular cells of the subject or organism. In one embodiment, the formulated molecular composition is contacted with the vascular or vascular cells of the subject or organism as is generally known in the art, such as via parental administration (e.g., intravenous, intramuscular, subcutaneous administration) or local administration (e.g., clamping, catheterization, stenting etc.) of the formulated molecular composition with or without excipients to facilitate the administration.

[0175] In one embodiment, the invention features a method for delivering or administering a biologically active molecule to skin or skin cells (e.g., dermis or dermis cells, follicle or follicular cells) in a subject or organism, comprising administering a formulated molecular composition of the invention under conditions suitable for delivery of the biologically active molecule component of the formulated molecular composition to the skin or skin cells of the subject or organism. In one embodiment, the formulated molecular composition is contacted with the skin or skin cells of the subject or organism as is generally known in the art, such as via parental administration (e.g., intravenous, intramuscular, subcutaneous administration) or local administration (e.g., direct dermal application, iontophoresis etc.) of the formulated molecular composition with or without excipients to facilitate the administration.

[0176] In one embodiment, the invention features a method for delivering or administering a biologically active molecule to the eye or ocular cells (e.g., macula, fovea, cornea, retina etc.) in a subject or organism, comprising administering a formulated molecular composition of the invention under conditions suitable for delivery of the

biologically active molecule component of the formulated molecular composition to the eye or ocular cells of the subject or organism. In one embodiment, the formulated molecular composition is contacted with the eye or ocular cells of the subject or organism as is generally known in the art, such as via parental administration (e.g., intravenous, intramuscular, subcutaneous administration) or local administration (e.g., direct injection, intraocular injection, periocular injection, iontophoresis, use of eyedrops, inplants etc.) of the formulated molecular composition with or without excipients to facilitate the administration.

[0177] In one embodiment, the invention features a method for delivering or administering a biologically active molecule to the ear or cells of the ear (e.g., inner ear, middle ear, outer ear) in a subject or organism, comprising administering a formulated molecular composition of the invention under conditions suitable for delivery of the biologically active molecule component of the formulated molecular composition to the ear or ear cells of the subject or organism. In one embodiment, the administration comprises methods and devices as described in U.S. Pat. Nos. 5,421,818, 5,476,446, 5,474,529, 6,045,528, 6,440,102, 6,685,697, 6,120,484; and 5,572,594; all incorporated by reference in their entireties herein and the teachings of Silverstein, 1999, Ear Nose Throat J., 78, 595-8, 600; and Jackson and Silverstein, 2002, Otolaryngol Clin North Am., 35, 639-53, and adapted for use the compositions of the invention.

[0178] In one embodiment, the invention features a formulated siNA composition comprising a short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene, wherein said siNA molecule comprises about 15 to about 28 base pairs.

[0179] In one embodiment, the invention features a formulated siNA composition comprising a double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a target RNA via RNA interference (RNAi), wherein the double stranded siNA molecule comprises a first and a second strand, each strand of the siNA molecule is about 18 to about 28 nucleotides in length, the first strand of the siNA comprises nucleotide sequence having sufficient complementarity to the target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference, and the second strand of said siNA molecule comprises nucleotide sequence that is complementary to the first strand.

[0180] In one embodiment, the invention features a formulated siNA composition comprising a double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a target RNA via RNA interference (RNAi), wherein the double stranded siNA molecule comprises a first and a second strand, each strand of the siNA molecule is about 18 to about 23 nucleotides in length, the first strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference, and the second strand of said siNA molecule comprises nucleotide sequence that is complementary to the first strand.

[0181] In one embodiment, the invention features a formulated siNA composition comprising a chemically synthesized double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a target RNA via RNA interference (RNAi), wherein each strand of the siNA mol-

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ecule is about 18 to about 28 nucleotides in length; and one strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference.

[0182]　In one embodiment, the invention features a formulated siNA composition comprising a chemically synthesized double stranded short interfering nucleic acid (siNA) molecule that directs cleavage of a target RNA via RNA interference (RNAi), wherein each strand of the siNA molecule is about 18 to about 23 nucleotides in length; and one strand of the siNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the siNA molecule to direct cleavage of the target RNA via RNA interference.

[0183]　In one embodiment, the invention features a formulated siNA composition comprising a siNA molecule that down-regulates expression of a target gene, for example, wherein the target gene comprises target encoding sequence. In one embodiment, the invention features a siNA molecule that down-regulates expression of a target gene, for example, wherein the target gene comprises target non-coding sequence or regulatory elements involved in target gene expression.

[0184]　In one embodiment, a siNA of the invention is used to inhibit the expression of target genes or a target gene family, wherein the genes or gene family sequences share sequence homology. Such homologous sequences can be identified as is known in the art, for example using sequence alignments. siNA molecules can be designed to target such homologous sequences, for example using perfectly complementary sequences or by incorporating non-canonical base pairs, for example mismatches and/or wobble base pairs that can provide additional target sequences. In instances where mismatches are identified, non-canonical base pairs (for example, mismatches and/or wobble bases) can be used to generate siNA molecules that target more than one gene sequence. In a non-limiting example, non-canonical base pairs such as UU and CC base pairs are used to generate siNA molecules that are capable of targeting sequences for differing targets that share sequence homology. As such, one advantage of using siNAs of the invention is that a single siNA can be designed to include nucleic acid sequence that is complementary to the nucleotide sequence that is conserved between the homologous genes. In this approach, a single siNA can be used to inhibit expression of more than one gene instead of using more than one siNA molecule to target the different genes.

[0185]　In one embodiment, the invention features a formulated siNA composition comprising a siNA molecule having RNAi activity against a target RNA, wherein the siNA molecule comprises a sequence complementary to any RNA having target encoding sequence. Examples of siNA molecules suitable for the formulations described herein are provided in International Application Serial Number US 04/106390 (WO 05/19453), which is hereby incorporated by reference in its entirety. Chemical modifications as described in PCT/US 2004/106390 (WO 05/19453), U.S. Ser. No. 10/444,853, filed May 23, 2003 U.S. Ser. No. 10/923,536 filed Aug. 20, 2004, U.S. Ser. No. 11/234,730, filed Sep. 23, 2005 or U.S. Ser. No. 11/299,254, filed Dec. 8, 2005, all incorporated by reference in their entireties

herein, or otherwise described herein can be applied to any siNA construct of the invention. In another embodiment, a siNA molecule of the invention includes a nucleotide sequence that can interact with nucleotide sequence of a target gene and thereby mediate silencing of target gene expression, for example, wherein the siNA mediates regulation of target gene expression by cellular processes that modulate the chromatin structure or methylation patterns of the target gene and prevent transcription of the target gene.

[0186]　In one embodiment, siNA molecules of the invention are used to down regulate or inhibit the expression of target proteins arising from target haplotype polymorphisms that are associated with a disease or condition (e.g. alopecia, hair loss, and/or atrichia). Analysis of target genes, or target protein or RNA levels can be used to identify subjects with such polymorphisms or those subjects who are at risk of developing traits, conditions, or diseases described herein. These subjects are amenable to treatment, for example, treatment with siNA molecules of the invention and any other composition useful in treating diseases related to target gene expression. As such, analysis of target protein or RNA levels can be used to determine treatment type and the course of therapy in treating a subject. Monitoring of target protein or RNA levels can be used to predict treatment outcome and to determine the efficacy of compounds and compositions that modulate the level and/or activity of certain target proteins associated with a trait, condition, or disease.

[0187]　In one embodiment, a siNA molecule of the invention comprises an antisense strand comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a target protein. The siNA further comprises a sense strand, wherein said sense strand comprises a nucleotide sequence of a target gene or a portion thereof.

[0188]　In another embodiment, a siNA of the invention comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence encoding a target protein or a portion thereof. The siNA molecule further comprises a sense region, wherein said sense region comprises a nucleotide sequence of a target gene or a portion thereof.

[0189]　In another embodiment, a siNA of the invention comprises a nucleotide sequence in the antisense region of the siNA molecule that is complementary to a nucleotide sequence or portion of sequence of a target gene. In another embodiment, a siNA of the invention comprises a region, for example, the antisense region of the siNA construct that is complementary to a sequence comprising a target gene sequence or a portion thereof.

[0190]　In one embodiment, a siNA molecule of the invention comprises an antisense strand having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein the antisense strand is complementary to a RNA sequence or a portion thereof encoding a target protein, and wherein said siNA further comprises a sense strand having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, and wherein said sense strand and said antisense strand are distinct nucleotide sequences where at least about 15 nucleotides in each strand are complementary to the other strand.

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[0191] In another embodiment, a siNA molecule of the invention comprises an antisense region having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein the antisense region is complementary to a RNA sequence encoding a target protein, and wherein said siNA further comprises a sense region having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein said sense region and said antisense region are comprised in a linear molecule where the sense region comprises at least about 15 nucleotides that are complementary to the antisense region.

[0192] In one embodiment, a siNA molecule of the invention has RNAi activity that modulates expression of RNA encoded by a target gene. Because target genes can share some degree of sequence homology with each other, siNA molecules can be designed to target a class of target genes or alternately specific target genes (e.g., polymorphic variants) by selecting sequences that are either shared amongst different targets or alternatively that are unique for a specific target. Therefore, in one embodiment, the siNA molecule can be designed to target conserved regions of target RNA sequences having homology among several target gene variants so as to target a class of target genes with one siNA molecule. Accordingly, in one embodiment, the siNA molecule of the invention modulates the expression of one or both target alleles in a subject. In another embodiment, the siNA molecule can be designed to target a sequence that is unique to a specific target RNA sequence (e.g., a single target allele or target single nucleotide polymorphism (SNP)) due to the high degree of specificity that the siNA molecule requires to mediate RNAi activity.

[0193] In one embodiment, a siNA molecule of the invention is double-stranded. In another embodiment, the siNA molecules of the invention consist of duplex nucleic acid molecules containing about 15 to about 30 base pairs between oligonucleotides comprising about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides. In yet another embodiment, siNA molecules of the invention comprise duplex nucleic acid molecules with overhanging ends of about 1 to about 3 (e.g., about 1, 2, or 3) nucleotides, for example, about 21-nucleotide duplexes with about 19 base pairs and 3'-terminal mononucleotide, dinucleotide, or trinucleotide overhangs. In yet another embodiment, siNA molecules of the invention comprise duplex nucleic acid molecules with blunt ends, where both ends are blunt, or alternatively, where one of the ends is blunt.

[0194] In one embodiment, siNA molecules of the invention have specificity for nucleic acid molecules expressing target proteins, such as RNA encoding a target protein. In one embodiment, a siNA molecule of the invention is RNA based (e.g., a siNA comprising 2'-OH nucleotides) and includes one or more chemical modifications, such as those described herein. Non-limiting examples of such chemical modifications include without limitation phosphorothioate internucleotide linkages, 2'-deoxyribonucleotides, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, "universal base" nucleotides, "acyclic" nucleotides, 5-C-methyl nucleotides, and terminal glyceryl and/or inverted deoxy abasic residue incorporation. These chemical modifications, when used in various siNA constructs, (e.g., RNA based siNA constructs), are shown to preserve RNAi activity

in cells while at the same time, dramatically increasing the serum stability of these compounds. Furthermore, contrary to the data published by Parrish et al., supra, applicant demonstrates that multiple (greater than one) phosphorothioate substitutions are well-tolerated and confer substantial increases in serum stability for modified siNA constructs.

[0195] In one embodiment, a siNA molecule of the invention comprises modified nucleotides while maintaining the ability to mediate RNAi. The modified nucleotides can be used to improve in vitro or in vivo characteristics such as stability, activity, and/or bioavailability. For example, a siNA molecule of the invention can comprise modified nucleotides as a percentage of the total number of nucleotides present in the siNA molecule. As such, a siNA molecule of the invention can generally comprise about 5% to about 100% modified nucleotides (e.g., about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides). The actual percentage of modified nucleotides present in a given siNA molecule will depend on the total number of nucleotides present in the siNA. If the siNA molecule is single stranded, the percent modification can be based upon the total number of nucleotides present in the single stranded siNA molecules. Likewise, if the siNA molecule is double stranded, the percent modification can be based upon the total number of nucleotides present in the sense strand, antisense strand, or both the sense and antisense strands.

[0196] One aspect of the invention features a formulated siNA composition comprising a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene. In one embodiment, the double stranded siNA molecule comprises one or more chemical modifications and each strand of the double-stranded siNA is about 21 nucleotides long. In one embodiment, the double-stranded siNA molecule does not contain any ribonucleotides. In another embodiment, the double-stranded siNA molecule comprises one or more ribonucleotides. In one embodiment, each strand of the double-stranded siNA molecule independently comprises about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein each strand comprises about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to the nucleotides of the other strand. In one embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of the target gene, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence of the target gene or a portion thereof.

[0197] In another embodiment, the invention features a formulated siNA composition comprising a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene comprising an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of the target gene or a portion thereof, and a sense region, wherein the sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence of the target gene or a portion thereof. In one embodiment, the

antisense region and the sense region independently comprise about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, wherein the antisense region comprises about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to nucleotides of the sense region.

[0198] In another embodiment, the invention features a formulated siNA composition comprising a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the target gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region.

[0199] In one embodiment, a siNA molecule of the invention comprises blunt ends, i.e., ends that do not include any overhanging nucleotides. For example, a siNA molecule comprising modifications described in U.S. Ser. No. 10/444,853, filed May 23, 2003, U.S. Ser. No. 10/923,536 filed Aug. 20, 2004, or U.S. Ser. No. 11/234,730, filed Sep. 23, 2005, all incorporated by reference in their entireties herein, or any combination thereof and/or any length described herein can comprise blunt ends or ends with no overhanging nucleotides.

[0200] In one embodiment, any siNA molecule of the invention can comprise one or more blunt ends, i.e. where a blunt end does not have any overhanging nucleotides. In one embodiment, the blunt ended siNA molecule has a number of base pairs equal to the number of nucleotides present in each strand of the siNA molecule. In another embodiment, the siNA molecule comprises one blunt end, for example wherein the 5'-end of the antisense strand and the 3'-end of the sense strand do not have any overhanging nucleotides. In another example, the siNA molecule comprises one blunt end, for example wherein the 3'-end of the antisense strand and the 5'-end of the sense strand do not have any overhanging nucleotides. In another example, a siNA molecule comprises two blunt ends, for example wherein the 3'-end of the antisense strand and the 5'-end of the sense strand as well as the 5'-end of the antisense strand and 3'-end of the sense strand do not have any overhanging nucleotides. A blunt ended siNA molecule can comprise, for example, from about 15 to about 30 nucleotides (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides). Other nucleotides present in a blunt ended siNA molecule can comprise, for example, mismatches, bulges, loops, or wobble base pairs to modulate the activity of the siNA molecule to mediate RNA interference.

[0201] By "blunt ends" is meant symmetric termini, or termini of a double stranded siNA molecule having no overhanging nucleotides. The two strands of a double stranded siNA molecule align with each other without over-hanging nucleotides at the termini. For example, a blunt ended siNA construct comprises terminal nucleotides that are complementary between the sense and antisense regions of the siNA molecule.

[0202] In one embodiment, the invention features a formulated siNA composition comprising a double-stranded short interfering nucleic acid (siNA) molecule that down-

regulates expression of a target gene, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. The sense region can be connected to the antisense region via a linker molecule, such as a polynucleotide linker or a non-nucleotide linker.

[0203] In one embodiment, the invention features a formulated siNA composition comprising a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene, wherein the siNA molecule comprises about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein each strand of the siNA molecule comprises one or more chemical modifications. In another embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a target gene or a portion thereof, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of the target gene. In another embodiment, one of the strands of the double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence of a target gene or portion thereof, and the second strand of the double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or portion thereof of the target gene. In another embodiment, each strand of the siNA molecule comprises about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides, and each strand comprises at least about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to the nucleotides of the other strand.

[0204] In any of the embodiments described herein, a siNA molecule of the invention can comprise no ribonucleotides. Alternatively, a siNA molecule of the invention can comprise one or more ribonucleotides.

[0205] In one embodiment, a siNA molecule of the invention comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence of a target gene or a portion thereof, and the siNA further comprises a sense region comprising a nucleotide sequence substantially similar to the nucleotide sequence of the target gene or a portion thereof. In another embodiment, the antisense region and the sense region each comprise about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides and the antisense region comprises at least about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides that are complementary to nucleotides of the sense region. The target gene can comprise, for example, sequences referred to by Genbank Accession Nos. in PCT Publication No. WO 03/74654, serial No. PCT/US03/05028. In another embodiment, the siNA is a double stranded nucleic acid molecule, where each of the two strands of the siNA molecule independently comprise about 15 to about 40 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 23, 33, 34, 35, 36, 37, 38, 39, or 40) nucleotides, and where one of the strands of the siNA molecule comprises at least about 15 (e.g. about 15, 16, 17,

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18, 19, 20, 21, 22, 23, 24 or 25 or more) nucleotides that are complementary to the nucleic acid sequence of the target gene or a portion thereof.

[0206] In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by a target gene, or a portion thereof, and the sense region comprises a nucleotide sequence that is complementary to the antisense region. In one embodiment, the siNA molecule is assembled from two separate oligonucleotide fragments, wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. In another embodiment, the sense region is connected to the antisense region via a linker molecule. In another embodiment, the sense region is connected to the antisense region via a linker molecule, such as a nucleotide or non-nucleotide linker. The target gene can comprise, for example, sequences referred to in PCT Publication No. WO 03/74654, serial No. PCT/US03/05028 or U.S. Ser. No. 10/923,536 or otherwise known in the art.

[0207] In one embodiment, the invention features a formulated siNA composition comprising a double-stranded short interfering nucleic acid (siNA) molecule that downregulates expression of a target gene comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the target gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region, and wherein the siNA molecule has one or more modified pyrimidine and/or purine nucleotides. In one embodiment, the pyrimidine nucleotides in the sense region are 2'-O-methylpyrimidine nucleotides or 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In another embodiment, the pyrimidine nucleotides in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides. In another embodiment, the pyrimidine nucleotides in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides. In one embodiment, the pyrimidine nucleotides in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides present in the antisense region are 2'-O-methyl or 2'-deoxy purine nucleotides. In another embodiment of any of the above-described siNA molecules, any nucleotides present in a non-complementary region of the sense strand (e.g. overhang region) are 2'-deoxy nucleotides.

[0208] In one embodiment, the invention features a formulated siNA composition comprising a double-stranded short interfering nucleic acid (siNA) molecule that downregulates expression of a target gene, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule, and wherein the fragment comprising the sense region includes a terminal cap moiety at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the fragment. In one embodiment, the terminal cap moiety is an inverted deoxy abasic moiety or glyceryl moiety. In one embodiment, each of the two fragments of the siNA molecule indepen-

dently comprise about 15 to about 30 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides. In another embodiment, each of the two fragments of the siNA molecule independently comprise about 15 to about 40 (e.g. about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 23, 33, 34, 35, 36, 37, 38, 39, or 40) nucleotides. In a non-limiting example, each of the two fragments of the siNA molecule comprises about 21 nucleotides.

[0209] In one embodiment, the invention features a formulated siNA composition comprising a siNA molecule comprising at least one modified nucleotide, wherein the modified nucleotide is a 2'-deoxy-2'-fluoro nucleotide. The siNA can be, for example, about 15 to about 40 nucleotides in length. In one embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In one embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoro cytidine or 2'-deoxy-2'-fluoro uridine nucleotide. In another embodiment, the modified nucleotides in the siNA include at least one 2'-fluoro cytidine and at least one 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro cytidine nucleotides. In one embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro adenosine nucleotides. In one embodiment, all guanosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro guanosine nucleotides. The siNA can further comprise at least one modified internucleotidic linkage, such as phosphorothioate linkage. In one embodiment, the 2'-deoxy-2'-fluoronucleotides are present at specifically selected locations in the siNA that are sensitive to cleavage by ribonucleases, such as locations having pyrimidine nucleotides.

[0210] In one embodiment, the invention features a method of increasing the stability of a siNA molecule of the invention against cleavage by ribonucleases comprising introducing at least one modified nucleotide into the siNA molecule, wherein the modified nucleotide is a 2'-deoxy-2'-fluoro nucleotide. In one embodiment, all pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides. In one embodiment, the modified nucleotides in the siNA include at least one 2'-deoxy-2'-fluoro cytidine or 2'-deoxy-2'-fluoro uridine nucleotide. In another embodiment, the modified nucleotides in the siNA include at least one 2'-fluoro cytidine and at least one 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all uridine nucleotides present in the siNA are 2'-deoxy-2'-fluoro uridine nucleotides. In one embodiment, all cytidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro cytidine nucleotides. In one embodiment, all adenosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro adenosine nucleotides. In one embodiment, all guanosine nucleotides present in the siNA are 2'-deoxy-2'-fluoro guanosine nucleotides. The siNA can further comprise at least one modified internucleotidic linkage, such as phosphorothioate linkage. In one embodiment, the 2'-deoxy-2'-fluoronucleotides are present at specifically selected locations in the siNA that are sensitive to cleavage by ribonucleases, such as locations having pyrimidine nucleotides.

[0211] In one embodiment, the invention features a formulated siNA composition comprising a double-stranded

short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene comprising a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence of RNA encoded by the target gene or a portion thereof and the sense region comprises a nucleotide sequence that is complementary to the antisense region, and wherein the purine nucleotides present in the antisense region comprise 2'-deoxy-purine nucleotides. In an alternative embodiment, the purine nucleotides present in the antisense region comprise 2'-O-methyl purine nucleotides. In either of the above embodiments, the antisense region can comprise a phosphorothioate internucleotide linkage at the 3' end of the antisense region. Alternatively, in either of the above embodiments, the antisense region can comprise a glyceryl modification at the 3' end of the antisense region. In another embodiment of any of the above-described siNA molecules, any nucleotides present in a non-complementary region of the antisense strand (e.g. overhang region) are 2'-deoxy nucleotides.

[0212] In one embodiment, the antisense region of a siNA molecule of the invention comprises sequence complementary to a portion of a target transcript having sequence unique to a particular target disease related allele, such as sequence comprising a single nucleotide polymorphism (SNP) associated with the disease specific allele. As such, the antisense region of a siNA molecule of the invention can comprise sequence complementary to sequences that are unique to a particular allele to provide specificity in mediating selective RNAi against the disease, condition, or trait related allele.

[0213] In one embodiment, the invention features a formulated siNA composition comprising a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene, wherein the siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule, where each strand is about 21 nucleotides long and where about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule, wherein at least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule, where each strand is about 19 nucleotide long and where the nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule to form at least about 15 (e.g., 15, 16, 17, 18, or 19) base pairs, wherein one or both ends of the siNA molecule are blunt ends. In one embodiment, each of the two 3' terminal nucleotides of each fragment of the siNA molecule is a 2'-deoxy-pyrimidine nucleotide, such as a 2'-deoxy-thymidine. In another embodiment, all nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule. In another embodiment, the siNA molecule is a double stranded nucleic acid molecule of about 19 to about 25 base pairs having a sense region and an antisense region, where about 19 nucleotides of the antisense region are base-paired to the

nucleotide sequence or a portion thereof of the RNA encoded by the target gene. In another embodiment, about 21 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the target gene. In any of the above embodiments, the 5'-end of the fragment comprising said antisense region can optionally include a phosphate group.

[0214] In any of the embodiments described herein, a siNA molecule of the invention can comprise one or more of the stabilization chemistries shown in Table I or described in PCT/US 2004/106390 (WO 05/19453), U.S. Ser. No. 10/444,853, filed May 23, 2003 U.S. Ser. No. 10/923,536 filed Aug. 20, 2004, U.S. Ser. No. 11/234,730, filed Sep. 23, 2005 or U.S. Ser. No. 11/299,254, filed Dec. 8, 2005, all incorporated by reference in their entireties herein.

[0215] In one embodiment, the invention features a formulated siNA composition comprising a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target RNA sequence (e.g., wherein said target RNA sequence is encoded by a target gene involved in the target pathway), wherein the siNA molecule does not contain any ribonucleotides and wherein each strand of the double-stranded siNA molecule is about 15 to about 30 nucleotides. In one embodiment, the siNA molecule is 21 nucleotides in length. Examples of non-ribonucleotide containing siNA constructs are combinations of stabilization chemistries described in PCT/US 2004/106390 (WO 05/19453), U.S. Ser. No. 10/444,853, filed May 23, 2003 U.S. Ser. No. 10/923,536 filed Aug. 20, 2004, U.S. Ser. No. 11/234,730, filed Sep. 23, 2005 or U.S. Ser. No. 11/299,254, filed Dec. 8, 2005, all incorporated by reference in their entireties herein.

[0216] In one embodiment, the invention features a formulated siNA composition comprising a chemically synthesized double stranded RNA molecule that directs cleavage of a target RNA via RNA interference, wherein each strand of said RNA molecule is about 15 to about 30 nucleotides in length; one strand of the RNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the RNA molecule to direct cleavage of the target RNA via RNA interference; and wherein at least one strand of the RNA molecule optionally comprises one or more chemically modified nucleotides described herein, such as without limitation deoxynucleotides, 2'-O-methyl nucleotides, 2'-deoxy-2'-fluoro nucleotides, 2'-O-methoxy-ethyl nucleotides etc.

[0217] In one embodiment, the invention features a composition comprising a formulated siNA composition of the invention in a pharmaceutically acceptable carrier or diluent.

[0218] In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target RNA sequence, wherein the siNA molecule does not contain any ribonucleotides and wherein each strand of the double-stranded siNA molecule is about 15 to about 30 nucleotides. In one embodiment, the siNA molecule is 21 nucleotides in length. Examples of non-ribonucleotide containing siNA constructs are combinations of stabilization chemistries shown in Table I in any combination of Sense/Antisense chemistries, such as Stab 7/8, Stab 7/11, Stab 8/8, Stab 18/8, Stab 18/11, Stab 12/13, Stab 7/13, Stab 18/13, Stab 7/19, Stab 8/19, Stab 18/19, Stab 7/20, Stab 8/20, Stab 18/20, Stab 7/32, Stab

8/32, or Stab 18/32 (e.g., any siNA having Stab 7, 8, 11, 12, 13, 14, 15, 17, 18, 19, 20, or 32 sense or antisense strands or any combination thereof). Herein, numeric Stab chemistries can include both 2'-fluoro and 2'-OCF3 versions of the chemistries shown in Table I. For example, "Stab 7/8" refers to both Stab 7/8 and Stab 7F/8F etc. In one embodiment, the invention features a chemically synthesized double stranded RNA molecule that directs cleavage of a target RNA via RNA interference, wherein each strand of said RNA molecule is about 15 to about 30 nucleotides in length; one strand of the RNA molecule comprises nucleotide sequence having sufficient complementarity to the target RNA for the RNA molecule to direct cleavage of the target RNA via RNA interference; and wherein at least one strand of the RNA molecule optionally comprises one or more chemically modified nucleotides described herein, such as without limitation deoxynucleotides, 2'-O-methyl nucleotides, 2'-deoxy-2'-fluoro nucleotides, 2'-O-methoxyethyl nucleotides, 4'-thio nucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides, etc.

[0219] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein the chemical modification comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides comprising a backbone modified internucleotide linkage having Formula 1:

$$R_1 - X - \overset{\overset{Z}{\|}}{\underset{\underset{W}{|}}{P}} - Y - R_2$$
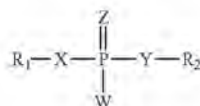
[0220] wherein each R1 and R2 is independently any nucleotide, non-nucleotide, or polynucleotide which can be naturally-occurring or chemically-modified, each X and Y is independently O, S, N, alkyl, or substituted alkyl, each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, aralkyl, or acetyl and wherein W, X, Y, and Z are optionally not all O. In another embodiment, a backbone modification of the invention comprises a phosphonoacetate and/or thiophosphonoacetate internucleotide linkage (see for example Sheehan et al., 2003, Nucleic Acids Research, 31, 4109-4118).

[0221] The chemically-modified internucleotide linkages having Formula I, for example, wherein any Z, W, X, and/or Y independently comprises a sulphur atom, can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) chemically-modified internucleotide linkages having Formula I at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified internucleotide linkages having Formula I at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can

comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In another embodiment, a siNA molecule of the invention having internucleotide linkage(s) of Formula I also comprises a chemically-modified nucleotide or non-nucleotide having any of Formulae I-VII.

[0222] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein the chemical modification comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula II:

wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I or II; R9 is O, S, CH2, S=O, CHF, or CF2, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA. In one embodiment, R3 and/or R7 comprises a conjugate moiety and a linker (e.g., a nucleotide or non-nucleotide linker as described herein or otherwise known in the art). Non-limiting examples of conjugate moieties include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetyl-galactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine.

[0223] The chemically-modified nucleotide or non-nucleotide of Formula II can be present in one or both oligonucleotide strands of the siNA duplex, for example in the

sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotides or non-nucleotides of Formula II at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 5'-end of the sense strand, the antisense strand, or both strands. In anther non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 3'-end of the sense strand, the antisense strand, or both strands.

[0224] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein the chemical modification comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula III:

$$R_7\!-\!\!-\!R_{11}$$
$$R_{12}\quad R_9\quad R_{10}$$
$$R_6\quad R_4$$
$$R_8\quad B$$
$$R_5\quad R_3$$

wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I or II; R9 is O, S, CH2, S=O, CHF, or CF2, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be employed to be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA. In one embodiment, R3 and/or R7 comprises a conjugate moiety and a linker (e.g., a nucleotide or non-nucleotide linker as described herein or otherwise known in the art). Non-limiting examples of conjugate moieties include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine.

[0225] The chemically-modified nucleotide or non-nucleotide of Formula III can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotides or non-nucleotides of Formula III at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide(s) or non-nucleotide(s) of Formula III at the 5'-end of the sense strand, the antisense strand, or both strands. In anther non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end of the sense strand, the antisense strand, or both strands.

[0226] In another embodiment, a siNA molecule of the invention comprises a nucleotide having Formula II or III, wherein the nucleotide having Formula II or III is in an inverted configuration. For example, the nucleotide having Formula II or III is connected to the siNA construct in a 3'-3',3'-2',2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

[0227] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein the chemical modification comprises a 5'-terminal phosphate group having Formula IV:

$$X\!-\!\!\overset{\overset{\displaystyle Z}{\|}}{\underset{\displaystyle W}{P}}\!-\!Y\!-\!$$
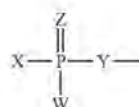
wherein each X and Y is independently O, S, N, alkyl, substituted alkyl, or alkylhalo; wherein each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, aralkyl, alkylhalo, or acetyl; and wherein W, X, Y and Z are not all O.

[0228] In one embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand, for example, a strand complementary to a target RNA, wherein the siNA molecule comprises an all RNA siNA molecule. In another embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand wherein the siNA molecule also comprises about 1 to about 3 (e.g., about 1, 2, or 3) nucleotide 3'-terminal nucleotide overhangs having about 1 to about 4 (e.g., about 1, 2, 3, or 4) deoxyribonucleotides on the 3'-end of one or both strands. In another embodiment, a 5'-terminal phosphate group having Formula IV is present on the target-complementary strand of a siNA molecule of the invention, for example a siNA molecule having chemical modifications having any of Formulae I-VII.

[0229] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) mol-

ecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein the chemical modification comprises one or more phosphorothioate internucleotide linkages. For example, in a non-limiting example, the invention features a chemically-modified short interfering nucleic acid (siNA) having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in one siNA strand. In yet another embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) individually having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in both siNA strands. The phosphorothioate internucleotide linkages can be present in one or both oligonucleotide strands of the siNA duplex, for example i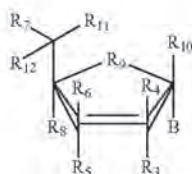n the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more phosphorothioate internucleotide linkages at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) consecutive phosphorothioate internucleotide linkages at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands.

[0230] In one embodiment, the invention features a siNA molecule, wherein the sense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy and/or about one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

[0231] In another embodiment, the invention features a siNA molecule, wherein the sense strand comprises about 1 to about 5, specifically about 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (e.g., about 1, 2, 3, 4, 5, or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5 or more, for example about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

[0232] In one embodiment, the invention features a siNA molecule, wherein the antisense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or about one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3' and 5'-ends, being present in the same or different strand.

[0233] In another embodiment, the invention features a siNA molecule, wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or

more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluorom-ethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothio-ate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluo-romethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a termi-nal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy, 4'-thio and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5, for example about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

[0234] In one embodiment, the invention features a chemi-cally-modified short interfering nucleic acid (siNA) mol-ecule having about 1 to about 5 or more (specifically about 1, 2, 3, 4, 5 or more) phosphorothioate internucleotide linkages in each strand of the siNA molecule.

[0235] In another embodiment, the invention features a siNA molecule comprising 2'-5' internucleotide linkages. The 2'-5' internucleotide linkage(s) can be at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of one or both siNA sequence strands. In addition, the 2'-5' internucleotide link-age(s) can be present at various other positions within one or both siNA sequence strands, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a pyrimidine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a purine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage.

[0236] In another embodiment, a chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified, wherein each strand is independently about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length, wherein the duplex has about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein the chemical modification comprises a structure having any of Formulae I-VII. For example, an exemplary chemically-modified siNA molecule of the invention com-prises a duplex having two strands, one or both of which can be chemically-modified with a chemical modification hav-ing any of Formulae I-VII or any combination thereof, wherein each strand consists of about 21 nucleotides, each having a 2-nucleotide 3'-terminal nucleotide overhang, and

wherein the duplex has about 19 base pairs. In another embodiment, a siNA molecule of the invention comprises a single stranded hairpin structure, wherein the siNA is about 36 to about 70 (e.g., about 36, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein the siNA can include a chemical modification comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 42 to about 50 (e.g., about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combi-nation thereof, wherein the linear oligonucleotide forms a hairpin structure having about 19 to about 21 (e.g., 19, 20, or 21) base pairs and a 2-nucleotide 3'-terminal nucleotide overhang. In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegrad-able. For example, a linear hairpin siNA molecule of the invention is designed such that degradation of the loop portion of the siNA molecule in vivo can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucle-otides.

[0237] In another embodiment, a siNA molecule of the invention comprises a hairpin structure, wherein the siNA is about 25 to about 50 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides in length having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemi-cally-modified siNA molecule of the invention comprises a linear oligonucleotide having about 25 to about 35 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35) nucleotides that is chemically-modified with one or more chemical modifications having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs and a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV). In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegrad-able. In one embodiment, a linear hairpin siNA molecule of the invention comprises a loop portion comprising a non-nucleotide linker.

[0238] In another embodiment, a siNA molecule of the invention comprises an asymmetric hairpin structure, wherein the siNA is about 25 to about 50 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides in length having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the

invention comprises a linear oligonucleotide having about 25 to about 35 (e.g., about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35) nucleotides that is chemically-modified with one or more chemical modifications having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms an asymmetric hairpin structure having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs and a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV). In one embodiment, an asymmetric hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. In another embodiment, an asymmetric hairpin siNA molecule of the invention comprises a loop portion comprising a non-nucleotide linker.

[0239] In another embodiment, a siNA molecule of the invention comprises an asymmetric double stranded structure having separate polynucleotide strands comprising sense and antisense regions, wherein the antisense region is about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides in length, wherein the sense region is about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides in length, wherein the sense region and the antisense region have at least 3 complementary nucleotides, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises an asymmetric double stranded structure having separate polynucleotide strands comprising sense and antisense regions, wherein the antisense region is about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) nucleotides in length and wherein the sense region is about 3 to about 15 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15) nucleotides in length, wherein the sense region the antisense region have at least 3 complementary nucleotides, and wherein the siNA can include one or more chemical modifications comprising a structure having any of Formulae I-VII or any combination thereof. In another embodiment, the asymmetric double stranded siNA molecule can also have a 5'-terminal phosphate group that can be chemically modified as described herein (for example a 5'-terminal phosphate group having Formula IV).

[0240] In another embodiment, a siNA molecule of the invention comprises a circular nucleic acid molecule, wherein the siNA is about 38 to about 70 (e.g., about 38, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) base pairs, and wherein the siNA can include a chemical modification, which comprises a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a circular oligonucleotide having about 42 to about 50 (e.g., about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the circular oligonucleotide forms a dumbbell shaped structure having about 19 base pairs and 2 loops.

[0241] In another embodiment, a circular siNA molecule of the invention contains two loop motifs, wherein one or

both loop portions of the siNA molecule is biodegradable. For example, a circular siNA molecule of the invention is designed such that degradation of the loop portions of the siNA molecule in vivo can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

[0242] In one embodiment, a siNA molecule of the invention comprises at least one (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) abasic moiety, for example a compound having Formula V:
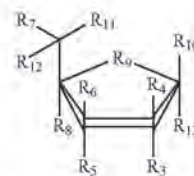
wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I or II; R9 is O, S, CH2, S=O, CHF, or CF2. In one embodiment, R3 and/or R7 comprises a conjugate moiety and a linker (e.g., a nucleotide or non-nucleotide linker as described herein or otherwise known in the art). Non-limiting examples of conjugate moieties include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetyl-galactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine.

[0243] In one embodiment, a siNA molecule of the invention comprises at least one (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) inverted abasic moiety, for example a compound having Formula VI:

wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2,

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N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I or II; R9 is O, S, CH2, S=O, CHF, or CF2, and either R2, R3, R8 or R13 serve as points of attachment to the siNA molecule of the invention. In one embodiment, R3 and/or R7 comprises a conjugate moiety and a linker (e.g., a nucleotide or non-nucleotide linker as described herein or otherwise known in the art). Non-limiting examples of conjugate moieties include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine.

[0244] In another embodiment, a siNA molecule of the invention comprises at least one (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) substituted polyalkyl moieties, for example a compound having Formula VII:
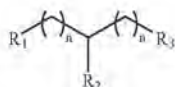
wherein each n is independently an integer from 1 to 12, each R1, R2 and R3 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or a group having Formula I, and R1, R2 or R3 serves as points of attachment to the siNA molecule of the invention. In one embodiment, R3 and/or R1 comprises a conjugate moiety and a linker (e.g., a nucleotide or non-nucleotide linker as described herein or otherwise known in the art). Non-limiting examples of conjugate moieties include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine.

[0245] By "ZIP code" sequences is meant, any peptide or protein sequence that is involved in cellular topogenic signaling mediated transport (see for example Ray et al., 2004, *Science*, 306(1501): 1505)

[0246] In another embodiment, the invention features a compound having Formula VII, wherein R1 and R2 are hydroxyl (OH) groups, n=1, and R3 comprises O and is the point of attachment to the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both strands of a double-stranded

siNA molecule of the invention or to a single-stranded siNA molecule of the invention. This modification is referred to herein as "glyceryl".

[0247] In another embodiment, a chemically modified nucleoside or non-nucleoside (e.g. a moiety having any of Formula V, VI or VII) of the invention is at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of a siNA molecule of the invention. For example, chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) can be present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense strand, the sense strand, or both antisense and sense strands of the siNA molecule. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the terminal position of the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the two terminal positions of the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In one embodiment, the chemically modified nucleoside or non-nucleoside (e.g., a moiety having Formula V, VI or VII) is present at the penultimate position of the 5'-end and 3'-end of the sense strand and the 3'-end of the antisense strand of a double stranded siNA molecule of the invention. In addition, a moiety having Formula VII can be present at the 3'-end or the 5'-end of a hairpin siNA molecule as described herein.

[0248] In another embodiment, a siNA molecule of the invention comprises an abasic residue having Formula V or VI, wherein the abasic residue having Formula VI or VI is connected to the siNA construct in a 3'-3',3'-2',2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

[0249] In one embodiment, a siNA molecule of the invention comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) locked nucleic acid (LNA) nucleotides, for example, at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

[0250] In one embodiment, a siNA molecule of the invention comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) 4'-thio nucleotides, for example, at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

[0251] In another embodiment, a siNA molecule of the invention comprises one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) acyclic nucleotides, for example, at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

[0252] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-

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2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

[0253] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

[0254] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the sense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides).

[0255] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising a sense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), wherein any (e.g., one or more or all) purine nucleotides present in the sense region are 2'-O-methyl, 4'-thio, 2'-O-

trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides), and wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

[0256] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides).

[0257] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides), and wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said antisense region are 2'-deoxy nucleotides.

[0258] In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region,

wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

[0259]   In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention comprising an antisense region, wherein any (e.g., one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any (e.g., one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides).

[0260]   In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system comprising a sense region, wherein one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and one or more purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), and an antisense region, wherein one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides or

alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and one or more purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides). The sense region and/or the antisense region can have a terminal cap modification that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense and/or antisense sequence. The sense and/or antisense region can optionally further comprise a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides. The overhang nucleotides can further comprise one or more (e.g., about 1, 2, 3, 4 or more) phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate internucleotide linkages. In any of these described embodiments, the purine nucleotides present in the sense region are alternatively 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides) and one or more purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides). Also, in any of these embodiments, one or more purine nucleotides present in the sense region are alternatively purine ribonucleotides (e.g., wherein all purine nucleotides are purine ribonucleotides or alternately a plurality of purine nucleotides are purine ribonucleotides) and any purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides). Additionally, in any of these embodiments, one or more purine nucleotides present in the sense region and/or present in the antisense region are alternatively selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides and 2'-O-methyl nucleotides

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(e.g., wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides and 2'-O-methyl nucleotides or alternately a plurality of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides and 2'-O-methyl nucleotides).

[0261] In another embodiment, any modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, are resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi. Non-limiting examples of nucleotides having a northern configuration include locked nucleic acid (LNA) nucleotides (e.g., 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides); 2'-methoxyethoxy (MOE) nucleotides; 2'-methyl-thio-ethyl, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy-2'-chloro nucleotides, 2'-azido nucleotides, 2'-O-trifluoromethyl nucleotides, 2'-O-ethyl-trifluoromethoxy nucleotides, 2'-O-difluoromethoxy-ethoxy nucleotides, 4'-thio nucleotides and 2'-O-methyl nucleotides.

[0262] In one embodiment, the sense strand of a double stranded siNA molecule of the invention comprises a terminal cap moiety, such as an inverted deoxyabasic moiety, at the 3'-end, 5'-end, or both 3' and 5'-ends of the sense strand.

[0263] In one embodiment, the invention features a chemically-modified short interfering nucleic acid molecule (siNA) capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein the chemical modification comprises a conjugate covalently attached to the chemically-modified siNA molecule. Non-limiting examples of conjugates contemplated by the invention include conjugates and ligands described in Vargeese et al., U.S. Ser. No. 10/427,160, filed Apr. 30, 2003, incorporated by reference herein in its entirety, including the drawings. In another embodiment, the conjugate is covalently attached to the chemically-modified siNA molecule via a biodegradable linker. In one embodiment, the conjugate molecule is attached at the 3'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In another embodiment, the conjugate molecule is attached at the 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In yet another embodiment, the conjugate molecule is attached both the 3'-end and 5'-end of either the sense strand, the antisense strand, or both

strands of the chemically-modified siNA molecule, or any combination thereof. In one embodiment, a conjugate molecule of the invention comprises a molecule that facilitates delivery of a chemically-modified siNA molecule into a biological system, such as a cell. In another embodiment, the conjugate molecule attached to the chemically-modified siNA molecule is a ligand for a cellular receptor, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; cholesterol; steroids, and polyamines, such as PEI, spermine or spermidine. Examples of specific conjugate molecules contemplated by the instant invention that can be attached to chemically-modified siNA molecules are described in Vargeese et al., U.S. Ser. No. 10/201,394, filed Jul. 22, 2002 incorporated by reference in its entirety herein. The type of conjugates used and the extent of conjugation of siNA molecules of the invention can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of siNA constructs while at the same time maintaining the ability of the siNA to mediate RNAi activity. As such, one skilled in the art can screen siNA constructs that are modified with various conjugates to determine whether the siNA conjugate complex possesses improved properties while maintaining the ability to mediate RNAi, for example in animal models as are generally known in the art.

[0264] In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule of the invention, wherein the siNA further comprises a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker that joins the sense region of the siNA to the antisense region of the siNA. In one embodiment, a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker is used, for example, to attach a conjugate moiety to the siNA. In one embodiment, a nucleotide linker of the invention can be a linker of ≥ 2 nucleotides in length, for example about 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In another embodiment, the nucleotide linker can be a nucleic acid aptamer.

[0265] In yet another embodiment, a non-nucleotide linker of the invention comprises abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (e.g. polyethylene glycols such as those having between 2 and 100 ethylene glycol units). Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, 18:6353 and *Nucleic Acids Res.* 1987, 15:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, 113:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, 113:5109; Ma et al., *Nucleic Acids Res.* 1993, 21:2585 and *Biochemistry* 1993, 32:1751; Durand et al., *Nucleic Acids Res.* 1990, 18:6353; McCurdy et al., *Nucleosides & Nucleotides* 1991, 10:287; Jschke et al., *Tetrahedron Lett.* 1993, 34:301; Ono et al., *Biochemistry* 1991, 30:9914; Arnold et al, International Publication No. WO 89/02439; Usman et al., International Publication No. WO 95/06731; Dudycz et al., International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, 113:4000, all hereby incorporated by reference herein. A "non-nucleotide" further means any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining

bases to exhibit their enzymatic activity. The group or compound can be abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine, for example at the C1 position of the sugar.

[0266] In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein one or both strands of the siNA molecule that are assembled from two separate oligonucleotides do not comprise any ribonucleotides. For example, a siNA molecule can be assembled from a single oligonculeotide where the sense and antisense regions of the siNA comprise separate oligonucleotides that do not have any ribonucleotides (e.g., nucleotides having a 2'-OH group) present in the oligonucleotides. In another example, a siNA molecule can be assembled from a single oligonculeotide where the sense and antisense regions of the siNA are linked or circularized by a nucleotide or non-nucleotide linker as described herein, wherein the oligonucleotide does not have any ribonucleotides (e.g., nucleotides having a 2'-OH group) present in the oligonucleotide. Applicant has surprisingly found that the presense of ribonucleotides (e.g., nucleotides having a 2'-hydroxyl group) within the siNA molecule is not required or essential to support RNAi activity. As such, in one embodiment, all positions within the siNA can include chemically modified nucleotides and/or non-nucleotides such as nucleotides and or non-nucleotides having Formula I, II, III, IV, V, VI, or VII or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

[0267] In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system comprising a single stranded polynucleotide having complementarity to a target nucleic acid sequence. In another embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group. In another embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group and a 3'-terminal phosphate group (e.g., a 2',3'-cyclic phosphate). In another embodiment, the single stranded siNA molecule of the invention comprises about 15 to about 30 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) nucleotides. In yet another embodiment, the single stranded siNA molecule of the invention comprises one or more chemically modified nucleotides or non-nucleotides described herein. For example, all the positions within the siNA molecule can include chemically-modified nucleotides such as nucleotides having any of Formulae I-VII, or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

[0268] In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system comprising a single stranded polynucleotide having complementarity to a target nucleic acid sequence, wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimi-

dine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, or 2'-O-difluoromethoxy-ethoxy purine nucleotides), and a terminal cap modification that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The siNA optionally further comprises about 1 to about 4 or more (e.g., about 1, 2, 3, 4 or more) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, 4 or more) phosphorothioate, phosphonoacetate, and/or thiophosphonoacetate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group. In any of these embodiments, any purine nucleotides present in the antisense region are alternatively 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides). Also, in any of these embodiments, any purine nucleotides present in the siNA (i.e., purine nucleotides present in the sense and/or antisense region) can alternatively be locked nucleic acid (LNA) nucleotides (e.g., wherein all purine nucleotides are LNA nucleotides or alternately a plurality of purine nucleotides are LNA nucleotides). Also, in any of these embodiments, any purine nucleotides present in the siNA are alternatively 2'-methoxyethyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-methoxyethyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-methoxyethyl purine nucleotides). In another embodiment, any modified nucleotides present in the single stranded siNA molecules of the invention comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, Principles of Nucleic Acid Structure, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the single stranded siNA molecules of the invention are preferably resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi.

[0269] In one embodiment, a siNA molecule of the invention comprises chemically modified nucleotides or non-nucleotides (e.g., having any of Formulae I-VII, such as 2'-deoxy, 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy or 2'-O-methyl nucleotides) at alternating positions within one or more strands or regions of the siNA molecule. For example, such chemical modifications can be introduced at every other position of a RNA based siNA molecule, starting at either the first or second nucleotide from the 3'-end or 5'-end of the siNA. In a non-limiting example, a double stranded siNA molecule of the invention in which each

strand of the siNA is 21 nucleotides in length is featured wherein positions 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 of each strand are chemically modified (e.g., with compounds having any of Formulae I-VII, such as such as 2'-deoxy, 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy or 2'-O-methyl nucleotides). In another non-limiting example, a double stranded siNA molecule of the invention in which each strand of the siNA is 21 nucleotides in length is featured wherein positions 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 of each strand are chemically modified (e.g., with compounds having any of Formulae I-VII, such as such as 2'-deoxy, 2'-deoxy-2'-fluoro, 4'-thio, 2'-O-trifluoromethyl, 2'-O-ethyl-trifluoromethoxy, 2'-O-difluoromethoxy-ethoxy or 2'-O-methyl nucleotides). Such siNA molecules can further comprise terminal cap moieties and/or backbone modifications as described herein.

[0270] In one embodiment, the invention features a method for delivering or administering a biologically active molecule, such as a polynucleotide molecule (e.g., siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule) of the invention to a cell or cells in a subject or organism, comprising administering a formulated molecular composition of the invention under conditions suitable for delivery of the polynucleotide component of the formulated molecular composition to the cell or cells of the subject or organism. In separate embodiments, the cell is, for example, a lung cell, liver cell, CNS cell, PNS cell, tumor cell, kidney cell, vascular cell, skin cell, ocular cell, or cells of the ear.

[0271] In one embodiment, the invention features a method for delivering or administering a biologically active molecule, such as a polynucleotide molecule (e.g., siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule) of the invention to liver or liver cells (e.g., hepatocytes) in a subject or organism, comprising administering a formulated molecular composition of the invention under conditions suitable for delivery of the polynucleotide component of the formulated molecular composition to the liver or liver cells (e.g., hepatocytes) of the subject or organism.

[0272] In one embodiment, the invention features a method for modulating the expression of a target gene within a cell comprising, introducing a formulated molecular composition of the invention into a cell under conditions suitable to modulate the expression of the target gene in the cell. In one embodiment, the cell is a liver cell (e.g., hepatocyte). In other embodiments, the cell is, for example, a lung cell, CNS cell, PNS cell, tumor cell, kidney cell, vascular cell, skin cell, ocular cell, or cells of the ear. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule.

[0273] In another embodiment, the invention features a method for modulating the expression of more than one target gene within a cell comprising, introducing a formulated molecular composition of the invention into the cell under conditions suitable to modulate the expression of the target genes in the cell. In one embodiment, the cell is a liver cell (e.g., hepatocyte). In other embodiments, the cell is, for example, a lung cell, CNS cell, PNS cell, tumor cell, kidney

cell, vascular cell, skin cell, ocular cell, or cells of the ear. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule.

[0274] In one embodiment, the invention features a method for treating or preventing a disease, disorder, trait or condition related to gene expression in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the reduction of gene expression and thus reduction in the level of the respective protein/RNA relieves, to some extent, the symptoms of the disease, disorder, trait or condition.

[0275] In one embodiment, the invention features a method for treating or preventing cancer in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of cancer can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cancerous cells and tissues. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of cancer in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0276] In one embodiment, the invention features a method for treating or preventing a proliferative disease or condition in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the proliferative disease or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cells and tissues involved in proliferative disease. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or

subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the proliferative disease or condition in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0277] In one embodiment, the invention features a method for treating or preventing transplant and/or tissue rejection (allograft rejection) in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of transplant and/or tissue rejection (allograft rejection) can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cells and tissues involved in transplant and/or tissue rejection (allograft rejection). In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of transplant and/or tissue rejection (allograft rejection) in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0278] In one embodiment, the invention features a method for treating or preventing an autoimmune disease, disorder, trait or condition in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the autoimmune disease, disorder, trait or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cells and tissues involved in the autoimmune disease, disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the autoimmune disease, disorder, trait or condition in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0279] In one embodiment, the invention features a method for treating or preventing an infectious disease, disorder, trait or condition in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the infectious disease, disorder, trait or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cells and tissues involved in the infectious disease, disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the infectious disease, disorder, trait or condition in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0280] In one embodiment, the invention features a method for treating or preventing an age-related disease, disorder, trait or condition in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the age-related disease, disorder, trait or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cells and tissues involved in the age-related disease, disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the age-related disease, disorder, trait or condition in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0281] In one embodiment, the invention features a method for treating or preventing a neurologic or neurodegenerative disease, disorder, trait or condition in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the neurologic or neurodegenerative dis-

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ease, disorder, trait or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cells and tissues involved in the neurologic or neurodegenerative disease, disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via catheterization, osmotic pump administration (e.g., intrathecal or ventricular) intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the neurologic or neurodegenerative disease, disorder, trait or condition in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism. In one embodiment, the neurologic disease is Huntington disease.

[0282] In one embodiment, the invention features a method for treating or preventing a metabolic disease, disorder, trait or condition in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the metabolic disease, disorder, trait or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cells and tissues involved in the metabolic disease, disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the metabolic disease, disorder, trait or condition in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0283] In one embodiment, the invention features a method for treating or preventing a cardiovascular disease, disorder, trait or condition in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the cardiovascular disease, disorder, trait or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one

embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cells and tissues involved in the cardiovascular disease, disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the cardiovascular disease, disorder, trait or condition in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0284] In one embodiment, the invention features a method for treating or preventing a respiratory disease, disorder, trait or condition in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the respiratory disease, disorder, trait or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cells and tissues involved in the respiratory disease, disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the respiratory disease, disorder, trait or condition in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0285] In one embodiment, the invention features a method for treating or preventing an ocular disease, disorder, trait or condition in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the ocular disease, disorder, trait or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cells and tissues involved in the ocular disease, disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutane-

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ous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the ocular disease, disorder, trait or condition in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0286] In one embodiment, the invention features a method for treating or preventing a dermatological disease, disorder, trait or condition in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the dermatological disease, disorder, trait or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cells and tissues involved in the dermatological disease, disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the dermatological disease, disorder, trait or condition in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0287] In one embodiment, the invention features a method for treating or preventing a liver disease, disorder, trait or condition (e.g., hepatitis, HCV, HBV, diabetis, cirrhosis, hepatocellular carcinoma etc.) in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the liver disease, disorder, trait or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as liver cells and tissues involved in the liver disease, disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the liver disease, disorder, trait or condition in a subject or organism. The formulated molecular composition of the invention can

be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0288] In one embodiment, the invention features a method for treating or preventing a kidney/renal disease, disorder, trait or condition (e.g., polycystic kidney disease etc.) in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the kidney/renal disease, disorder, trait or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as kidney/renal cells and tissues involved in the kidney/renal disease, disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the kidney/renal disease, disorder, trait or condition in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0289] In one embodiment, the invention features a method for treating or preventing an auditory disease, disorder, trait or condition (e.g., hearing loss, deafness, etc.) in a subject or organism comprising contacting the subject or organism with a formulated molecular composition of the invention under conditions suitable to modulate the expression of the target gene in the subject or organism whereby the treatment or prevention of the auditory disease, disorder, trait or condition can be achieved. In one embodiment, the formulated molecular composition comprises a polynucleotide, such as a siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via local administration to relevant tissues or cells, such as cells and tissues of the ear, inner hear, or middle ear involved in the auditory disease, disorder, trait or condition. In one embodiment, the invention features contacting the subject or organism with a formulated molecular composition of the invention via systemic administration (such as via intravenous or subcutaneous administration of the formulated molecular composition) to relevant tissues or cells, such as tissues or cells involved in the maintenance or development of the auditory disease, disorder, trait or condition in a subject or organism. The formulated molecular composition of the invention can be formulated or conjugated as described herein or otherwise known in the art to target appropriate tisssues or cells in the subject or organism.

[0290] In one embodiment, the invention features a method for treating or preventing a disease or condition as

described herein in a subject or organism, comprising administering to the subject or organism a formulated molecular composition of the invention; wherein the formulated molecular composition is administered under conditions suitable for reducing or inhibiting the level of target gene expression in the subject compared to a subject not treated with the formulated molecular composition. In one embodiment, the formulated molecular composition comprises a lipid nanoparticle and a siNA molecule of the invention.

[0291] In one embodiment, the invention features a method for treating or preventing a disease or condition as described herein in a subject or organism, comprising administering to the subject a formulated molecular composition of the invention; wherein (a) the formulated moleculer composition comprises a double stranded nucleic acid molecule having a sense strand and an antisense strand; (b) each strand of the double stranded nucleic acid molecule is 15 to 28 nucleotides in length; (c) at least 15 nucleotides of the sense strand are complementary to the antisense strand (d) the antisense strand of the double stranded nucleic acid molecule has complementarity to a target RNA; and wherein the formulated molecular composition is administered under conditions suitable for reducing or inhibiting the target RNA in the subject compared to a subject not treated with the formulated molecular composition. In one embodiment, the formulated molecular composition comprises a lipid nanoparticle and a siNA molecule of the invention.

[0292] In one embodiment, the invention features a method for treating or preventing a disease or condition as described herein in a subject or organism, comprising administering to the subject a formulated molecular composition of the invention; wherein (a) the formulated moleculer composition comprises a double stranded nucleic acid molecule having a sense strand and an antisense strand; (b) each strand of the double stranded nucleic acid molecule is 15 to 28 nucleotides in length; (c) at least 15 nucleotides of the sense strand are complementary to the antisense strand (d) the antisense strand of the double stranded nucleic acid molecule has complementarity to a target RNA; (e) at least 20% of the internal nucleotides of each strand of the double stranded nucleic acid molecule are modified nucleosides having a chemical modification; and (f) at least two of the chemical modifications are different from each other, and wherein the formulated molecular composition is administered under conditions suitable for reducing or inhibiting the level of target RNA in the subject compared to a subject not treated with the formulated molecular composition. In one embodiment, the formulated molecular composition comprises a lipid nanoparticle and a siNA molecule of the invention.

[0293] In any of the methods of treatment of the invention, the formulated molecular composition can be administered to the subject as a course of treatment, for example administration at various time intervals, such as once per day over the course of treatment, once every two days over the course of treatment, once every three days over the course of treatment, once every four days over the course of treatment, once every five days over the course of treatment, once every six days over the course of treatment, once per week over the course of treatment, once every other week over the course of treatment, once per month over the course of treatment, etc. In one embodiment, the course of treatment

is once every 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 weeks. In one embodiment, the course of treatment is from about one to about 52 weeks or longer (e.g., indefinitely). In one embodiment, the course of treatment is from about one to about 48 months or longer (e.g., indefinitely).

[0294] In one embodiment, a course of treatment involves an initial course of treatment, such as once every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more weeks for a fixed interval (e.g., 1×, 2×, 3×, 4×, 5×, 6×, 7×, 8×, 9×, 10× or more) followed by a maintenance course of treatment, such as once every 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, or more weeks for an additional fixed interval (e.g., 1×, 2×, 3×, 4×, 5×, 6×, 7×, 8×, 9×, 10× or more).

[0295] In any of the methods of treatment of the invention, the formulated molecular composition can be administered to the subject systemically as described herein or otherwise known in the art. Systemic administration can include, for example, intravenous, subcutaneous, intramuscular, catheterization, nasopharangeal, transdermal, or gastrointestinal administration as is generally known in the art.

[0296] In one embodiment, in any of the methods of treatment or prevention of the invention, the formulated molecular composition can be administered to the subject locally or to local tissues as described herein or otherwise known in the art. Local administration can include, for example, catheterization, implantation, osmotic pumping, direct injection, dermal/transdermal application, stenting, ear/eye drops, or portal vein administration to relevant tissues, or any other local administration technique, method or procedure, as is generally known in the art.

[0297] In one embodiment, the invention features a composition comprising a formulated molecular composition of the invention, in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a pharmaceutical composition comprising formulated molecular compositions of the invention, targeting one or more genes in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a method for diagnosing a disease or condition in a subject comprising administering to the subject a formulated molecular composition of the invention under conditions suitable for the diagnosis of the disease or condition in the subject. In another embodiment, the invention features a method for treating or preventing a disease, trait, or condition in a subject, comprising administering to the subject a formulated molecular composition of the invention under conditions suitable for the treatment or prevention of the disease, trait or condition in the subject, alone or in conjunction with one or more other therapeutic compounds.

[0298] In one embodiment, the method of synthesis of polynucleotide molecules of the invention, including but not limited to siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecules, comprises the teachings of Scaringe et al., U.S. Pat. Nos. 5,889,136; 6,008,400; and 6,111,086, incorporated by reference herein in their entirety.

[0299] In another embodiment, the invention features a method for generating formulated polynucleotide (e.g., to siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule) compositions with increased nuclease resistance comprising

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(a) introducing modified nucleotides into a polynucleotide component of a formulated molecular composition of the invention, and (b) assaying the formulated molecular composition of step (a) under conditions suitable for isolating formulated polynucleotide compositions having increased nuclease resistance.

[0300] In another embodiment, the invention features a method for generating polynucleotide (e.g., to siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule) molecules with improved toxicologic profiles (e.g., having attenuated or no immunstimulatory properties) comprising (a) introducing nucleotides having any of Formula I-VII (e.g., siNA motifs referred to in Table I) or any combination thereof into a polynucleotide molecule, and (b) assaying the polynucleotide molecule of step (a) under conditions suitable for isolating siNA molecules having improved toxicologic profiles.

[0301] In another embodiment, the invention features a method for generating formulated siNA compositions with improved toxicologic profiles (e.g., having attenuated or no immunstimulatory properties) comprising (a) generating a formulated siNA composition comprising a siNA molecule of the invention and a delivery vehicle or delivery particle as described herein or as otherwise known in the art, and (b) assaying the siNA formualtion of step (a) under conditions suitable for isolating formulated siNA compositions having improved toxicologic profiles.

[0302] In another embodiment, the invention features a method for generating siNA molecules that do not stimulate an interferon response (e.g., no interferon response or attenuated interferon response) in a cell, subject, or organism, comprising (a) introducing nucleotides having any of Formula I-VII (e.g., siNA motifs referred to in Table I) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules that do not stimulate an interferon response.

[0303] In another embodiment, the invention features a method for generating formulated siNA compositions that do not stimulate an interferon response (e.g., no interferon response or attenuated interferon response) in a cell, subject, or organism, comprising (a) generating a formulated siNA composition comprising a siNA molecule of the invention and a delivery vehicle or delivery particle as described herein or as otherwise known in the art, and (b) assaying the siNA formualtion of step (a) under conditions suitable for isolating formulated siNA compositions that do not stimulate an interferon response. In one embodiment, the interferon comprises interferon alpha.

[0304] In another embodiment, the invention features a method for generating siNA molecules that do not stimulate an inflammatory or proinflammatory cytokine response (e.g., no cytokine response or attenuated cytokine response) in a cell, subject, or organism, comprising (a) introducing nucleotides having any of Formula I-VII (e.g., siNA motifs referred to in Table I) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules that do not stimulate a cytokine response. In one embodiment, the cytokine comprises an interleukin such as interleukin-6 (IL-6) and/or tumor necrosis factor alpha (TNF-α).

[0305] In another embodiment, the invention features a method for generating formulated siNA compositions that do not stimulate an inflammatory or proinflammatory cytokine response (e.g., no cytokine response or attenuated cytokine response) in a cell, subject, or organism, comprising (a) generating a formulated siNA composition comprising a siNA molecule of the invention and a delivery vehicle or delivery particle as described herein or as otherwise known in the art, and (b) assaying the siNA formualtion of step (a) under conditions suitable for isolating formulated siNA compositions that do not stimulate a cytokine response. In one embodiment, the cytokine comprises an interleukin such as interleukin-6 (IL-6) and/or tumor necrosis alpha (TNF-α).

[0306] In another embodiment, the invention features a method for generating siNA molecules that do not stimulate Toll-like Receptor (TLR) response (e.g., no TLR response or attenuated TLR response) in a cell, subject, or organism, comprising (a) introducing nucleotides having any of Formula I-VII (e.g., siNA motifs referred to in Table I) or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules that do not stimulate a TLR response. In one embodiment, the TLR comprises TLR3, TLR7, TLR8 and/or TLR9.

[0307] In another embodiment, the invention features a method for generating formulated siNA compositions that do not stimulate a Toll-like Receptor (TLR) response (e.g., no TLR response or attenuated TLR response) in a cell, subject, or organism, comprising (a) generating a formulated siNA composition comprising a siNA molecule of the invention and a delivery vehicle or delivery particle as described herein or as otherwise known in the art, and (b) assaying the siNA formualtion of step (a) under conditions suitable for isolating formulated siNA compositions that do not stimulate a TLR response. In one embodiment, the TLR comprises TLR3, TLR7, TLR8 and/or TLR9.

[0308] By "improved toxicologic profile", is meant that the polynucleotide, formulated molecular composition, siNA or formulated siNA composition exhibits decreased toxicity in a cell, subject, or organism compared to an unmodified polynucleotide, formulated molecular composition, siNA or formulated siNA composition, or siNA molecule having fewer modifications or modifications that are less effective in imparting improved toxicology. In a non-limiting example, polynucleotides, formulated molecular compositions, siNAs or formulated siNA compositions with improved toxicologic profiles are associated with reduced immunostimulatory properties, such as a reduced, decreased or attenuated immunostimulatory response in a cell, subject, or organism compared to an unmodified polynucleotide, formulated molecular composition, siNA or formulated siNA composition, or polynucleotide (e.g., siNA) molecule having fewer modifications or modifications that are less effective in imparting improved toxicology. Such an improved toxicologic profile is characterized by abrogated or reduced immunostimulation, such as reduction or abrogation of induction of interferons (e.g., interferon alpha), inflammatory cytokines (e.g., interleukins such as IL-6, and/or TNF-alpha), and/or toll like receptors (e.g., TLR-3, TLR-7, TLR-8, and/or TLR-9). In one embodiment, a polynucleotide, formulated molecular composition, siNA or formulated siNA composition with an improved toxicological profile comprises no ribonucleotides. In one embodiment, a

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polynucleotide, formulated molecular composition, siNA or formulated siNA composition with an improved toxicological profile comprises less than 5 ribonucleotides (e.g., 1, 2, 3, or 4 ribonucleotides). In one embodiment, a siNA or formulated siNA composition with an improved toxicological profile comprises Stab 7, Stab 8, Stab 11, Stab 12, Stab 13, Stab 16, Stab 17, Stab 18, Stab 19, Stab 20, Stab 23, Stab 24, Stab 25, Stab 26, Stab 27, Stab 28, Stab 29, Stab 30, Stab 31, Stab 32, Stab 33, Stab 34 or any combination thereof (see Table I). Herein, numeric Stab chemistries include both 2'-fluoro and 2'-OCF3 versions of the chemistries shown in Table IV. For example, "Stab 7/8" refers to both Stab 7/8 and Stab 7F/8F etc. In one embodiment, a siNA or formulated siNA composition with an improved toxicological profile comprises a siNA molecule as described in United States Patent Application Publication No. 20030077829, incorporated by reference herein in its entirety including the drawings.

[0309] In one embodiment, the level of immunostimulatory response associated with a given polynucleotide, formulated molecular composition, siNA molecule or formulated siNA composition can be measured as is described herein or as is otherwise known in the art, for example by determining the level of PKR/interferon response, proliferation, B-cell activation, and/or cytokine production in assays to quantitate the immunostimulatory response of particular polynucleotide molecules (see, for example, Leifer et al., 2003, *J Immunother.* 26, 313-9; and U.S. Pat. No. 5,968,909, incorporated in its entirety by reference). In one embodiment, the reduced immunostimulatory response is between about 10% and about 100% compared to an unmodified or minimally modified siRNA molecule, e.g., about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% reduced immunostimulatory response. In one embodiment, the immunostimulatory response associated with a siNA molecule can be modulated by the degree of chemical modification. For example, a siNA molecule having between about 10% and about 100%, e.g., about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the nucleotide positions in the siNA molecule modified can be selected to have a corresponding degree of immunostimulatory properties as described herein.

[0310] In one embodiment, the degree of reduced immunostimulatory response is selected for optimized RNAi activity. For example, retaining a certain degree of immunostimulation can be preferred to treat viral infection, where less than 100% reduction in immunostimulation may be preferred for maximal antiviral activity (e.g., about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% reduction in immunostimulation) whereas the inhibition of expression of an endogenous gene target may be preferred with siNA molecules that posess minimal immunostimulatory properties to prevent non-specific toxicity or off target effects (e.g., about 90% to about 100% reduction in immunostimulation).

[0311] In one embodiment, a formulated siNA composition of the invention is designed such that the composition is not toxic to cells or has a minimized toxicicological profile such that the composition does not interfere with the efficacy of RNAi mediated by the siNA component of the formulated siNA composition or result in toxicity to the cells.

[0312] The term "formulated molecular composition" or "lipid nanoparticle", or "lipid nanoparticle composition" or

"LNP as used herein refers to a composition comprising one or more biologically active molecules independently or in combination with a cationic lipid, a neutral lipid, and/or a polyethyleneglycol-diacylglycerol (i.e., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB) conjugate. A formulated molecular composition can further comprise cholesterol or a cholesterol derivative (see **FIG. 5**). The cationic lipid of the invention can comprise a compound having any of Formulae CLI, CLII, CLIII, CLIV, CLV, CLVI, CLVII, CLVIII, CLIX, CLX, CLXI, CLXII, CLXIII, CLXIV, CLXV, CLXVI, CLXVII, CLXVIII, CLXIX, CLXX, CLXXI, CLXXII, CLXXIII, CLXXIV, CLXXV, CLXXVI, CLXXVII, CLXXVIII, CLXXIX, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2, 3-dioleyloxy)propylamine (DODMA), 1,2-Dioleoyl-3-Dimethylammonium-propane (DODAP), 1,2-Dioleoylcarbamyl-3-Dimethylammonium-propane (DOCDAP), 1,2-Dilineoyl-3-Dimethylammonium-propane (DLINDAP), 3-Dimethylamino-2-(Cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',12'-octadecadienoxy)propane (CpLin DMA), N,N-Dimethyl-3,4-dioleyloxybenzylamine (DMOBA) and/or a mixture thereof. The neutral lipid can comprise dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC), cholesterol, and/or a mixture thereof. The PEG conjugate can comprise a PEG-dilaurylglycerol (C12), a PEG-dimyristylglycerol (C14), a PEG-dipalmitoylglycerol (C16), a PEG-disterylglycerol (C18), PEG-dilaurylglycamide (C12), PEG-dimyristylglycamide (C14), PEG-dipalmitoylglycamide (C16), PEG-disterylglycamide (C18), PEG-cholesterol, or PEG-DMB. The cationic lipid component can comprise from about 2% to about 60%, from about 5% to about 45%, from about 5% to about 15%, or from about 40% to about 50% of the total lipid present in the formulation. The neutral lipid component can comprise from about 5% to about 90%, or from about 20% to about 85% of the total lipid present in the formulation. The PEG-DAG conjugate (e.g., polyethyleneglycol diacylglycerol (PEG-DAG), PEG-cholesterol, or PEG-DMB) can comprise from about 1% to about 20%, or from about 4% to about 15% of the total lipid present in the formulation. The cholesterol component can comprise from about 10% to about 60%, or from about 20% to about 45% of the total lipid present in the formulation. In one embodiment, a formulated molecular composition of the invention comprises a cationic lipid component comprising about 7.5% of the total lipid present in the formulation, a neutral lipid comprising about 82.5% of the total lipid present in the formulation, and a PEG conjugate comprising about 10% of the total lipid present in the formulation. In one embodiment, a formulated molecular composition of the invention comprises a biologically active molecule, DODMA, DSPC, and a PEG-DAG conjugate. In one embodiment, the PEG-DAG conjugate is PEG-dilaurylglycerol (C12), PEG-dimyristylglycerol (C14), PEG-dipalmitoylglycerol (C16), or PEG-disterylglycerol (C18). In another embodiment, the formulated molecular composition also comprises cholesterol or a cholesterol derivative. In one embodiment, the

formulated molecular composition comprises a lipid nano-particle formulation as shown in Table IV.

[0313] The term "formulated siNA composition" as used herein refers to a composition comprising one or more siNA molecules or a vector encoding one or more siNA molecules independently or in combination with a cationic lipid, a neutral lipid, and/or a polyethyleneglycol-diacylglycerol (PEG-DAG) or PEG-cholesterol (PEG-Chol) conjugate. A formulated siNA composition can further comprise choles-terol or a cholesterol derivative. The cationic lipid of the invention can comprise a compound having any of Formulae CLI, CLII, CLIII, CLIV, CLV, CLVI, CLVII, CLVIII, CLIX, CLX, CLXI, CLXII, CLXIII, CLXIV, CLXV, CLXVI, CLXVII, CLXVIII, CLXIX, CLXX, CLXXI, CLXXII, CLXXIII, CLXXIV, CLXXV, CLXXVI, CLXXVII, CLXX-VIII, CLXXIX, N,N-dioleyl-N,N-dimethylammonium chlo-ride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(1-(2,3-dioley-loxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2,3-dioleyloxy)propylamine (DODMA), 1,2-Dioleoyl-3-Dimethylammonium-propane (DODAP), 1,2-Dioleoylcarbamyl-3-Dimethylammonium-propane (DOCDAP), 1,2-Dilineoyl-3-Dimethylammonium-propane (DLINDAP), 3-Dimethylamino-2-(Cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',12'-octadecadienoxy)propane (CpLin DMA), N,N-Dimethyl-3, 4-dioleyloxybenzylamine (DMOBA) and/or a mixture thereof. The neutral lipid can comprise a compound having any of Formulae NLI-NLVII, dioleoylphosphatidylethano-lamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), egg phosphatidylcholine (EPC), distearoylphos-phatidylcholine (DSPC), cholesterol, and/or a mixture thereof. The PEG conjugate can comprise a PEG-dilaurylg-lycerol (C12), a PEG-dimyristylglycerol (C14), a PEG-dipalmitoylglycerol (C16), a PEG-disterylglycerol (C18), PEG-dilaurylglycamide (C12), PEG-dimyristylglycamide (C14), PEG-dipalmitoylglycamide (C16), PEG-disterylgly-camide (C18), PEG-cholesterol, or PEG-DMB. The cationic lipid component can comprise from about 2% to about 60%, from about 5% to about 45%, from about 5% to about 15%, or from about 40% to about 50% of the total lipid present in the formulation. The neutral lipid component can comprise from about 5% to about 90%, or from about 20% to about 85% of the total lipid present in the formulation. The PEG-DAG conjugate can comprise from about 1% to about 20%, or from about 4% to about 15% of the total lipid present in the formulation. The cholesterol component can comprise from about 10% to about 60%, or from about 20% to about 45% of the total lipid present in the formulation. In one embodiment, a formulated siNA composition of the invention comprises a cationic lipid component comprising about 7.5% of the total lipid present in the formulation, a neutral lipid comprising about 82.5% of the total lipid present in the formulation, and a PEG-DAG conjugate comprising about 10% of the total lipid present in the formulation. In one embodiment, a formulated siNA com-position of the invention comprises a siNA molecule, DODMA, DSPC, and a PEG-DAG conjugate. In one embodiment, the PEG-DAG conjugate is PEG-dilaurylglyc-erol (C12), PEG-dimyristylglycerol (C14), PEG-dipalmi-toylglycerol (C16), or PEG-disterylglycerol (C18). In

another embodiment, the formulated siNA composition also comprises cholesterol or a cholesterol derivative.

[0314] By "cationic lipid" as used herein is meant any lipophilic compound having cationic change, such as a compound having any of Formulae CLI-CLXXIX.

[0315] By "neutral lipid" as used herein is meant any lipophilic compound having non-cationic change (e.g., anionic or neutral charge).

[0316] By "PEG" is meant, any polyethylene glycol or other polyalkylene ether or equivalent polymer.

[0317] By "nanoparticle" is meant a microscopic particle whose size is measured in nanometers. Nanoparticles of the invention typically range from about 1 to about 999 nm in diameter, and can include an encapsulated or enclosed biologically active molecule.

[0318] By "microparticle" is meant a is a microscopic particle whose size is measured in micrometers. Micropar-ticles of the invention typically range from about 1 to about 100 micrometers in diameter, and can include an encapsu-lated or enclosed biologically active molecule.

[0319] The terms "short interfering nucleic acid", "siNA", "short interfering RNA", "siRNA", "short interfering nucleic acid molecule", "short interfering oligonucleotide molecule", and "chemically-modified short interfering nucleic acid molecule" as used herein refer to any nucleic acid molecule capable of inhibiting or down regulating gene expression or viral replication by mediating RNA interfer-ence "RNAi" or gene silencing in a sequence-specific man-ner (see PCT/US 2004/106390 (WO 05/19453), U.S. Ser. No. 10/444,853, filed May 23, 2003 U.S. Ser. No. 10/923, 536 filed Aug. 20, 2004, U.S. Ser. No. 11/234,730, filed Sep. 23, 2005 or U.S. Ser. No. 11/299,254, filed Dec. 8, 2005, all incorporated by reference in their entireties herein). For example the siNA can be a double-stranded nucleic acid molecule comprising self-complementary sense and anti-sense regions, wherein the antisense region comprises nucle-otide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e., each strand comprises nucleotide sequence that is comple-mentary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure, for example wherein the double stranded region is about 15 to about 30, e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 base pairs; the antisense strand comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof (e.g., about 15 to about 25 or more nucleotides of the siNA molecule are complementary to the target nucleic acid or a portion thereof). Alternatively, the siNA is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the siNA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s). The

siNA can be a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either in vivo or in vitro to generate an active siNA molecule capable of mediating RNAi. The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (for example, where such siNA molecule does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example Martinez et al., 2002, Cell., 110, 563-574 and Schwarz et al., 2002, Molecular Cell, 10, 537-568), or 5',3'-diphosphate. In certain embodiments, the siNA molecule of the invention comprises separate sense and antisense sequences or regions, wherein the sense and antisense regions are covalently linked by nucleotide or non-nucleotide linkers molecules as is known in the art, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, van der waals interactions, hydrophobic interactions, and/or stacking interactions. In certain embodiments, the siNA molecules of the invention comprise nucleotide sequence that is complementary to nucleotide sequence of a target gene. In another embodiment, the siNA molecule of the invention interacts with nucleotide sequence of a target gene in a manner that causes inhibition of expression of the target gene. As used herein, siNA molecules need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. Applicant describes in certain embodiments short interfering nucleic acids that do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any ribonucleotides (e.g., nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions. The modified short interfering nucleic acid molecules of the invention can also be referred to as short interfering modified oligonucleotides "siMON." As used herein, the term siNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others. Non limiting examples of siNA molecules of the invention are shown in U.S. Ser. No. 11/234,730, filed Sep. 23, 2005, incorporated by reference in its entirety herein. Such siNA molecules are distinct from other nucleic acid technologies known in the art that mediate inhibition of gene expression, such as ribozymes, antisense, triplex forming, aptamer, 2,5-A chimera, or decoy oligonucleotides.

[0320] By "RNA interference" or "RNAi" is meant a biological process of inhibiting or down regulating gene expression in a cell as is generally known in the art and which is mediated by short interfering nucleic acid molecules, see for example Zamore and Haley, 2005, Science, 309, 1519-1524; Vaughn and Martienssen, 2005, Science, 309, 1525-1526; Zamore et al., 2000, Cell, 101, 25-33; Bass, 2001, Nature, 411, 428-429; Elbashir et al., 2001, Nature, 411, 494-498; and Kreutzer et al., International PCT Publication No. WO 00/44895; Zernicka-Goetz et al., International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck et al., International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li et al., International PCT Publication No. WO 00/44914; Allshire, 2002, Science, 297, 1818-1819; Volpe et al., 2002, Science, 297, 1833-1837; Jenuwein, 2002, Science, 297, 2215-2218; and Hall et al., 2002, Science, 297, 2232-2237; Hutvagner and Zamore, 2002, Science, 297, 2056-60; McManus et al., 2002, RNA, 8, 842-850; Reinhart et al., 2002, Gene & Dev., 16, 1616-1626; and Reinhart & Bartel, 2002, Science, 297, 1831). In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, translational inhibition, transcriptional inhibition, or epigenetics. For example, siNA molecules of the invention can be used to epigenetically silence genes at both the post-transcriptional level or the pre-transcriptional level. In a non-limiting example, epigenetic modulation of gene expression by siNA molecules of the invention can result from siNA mediated modification of chromatin structure or methylation patterns to alter gene expression (see, for example, Verdel et al., 2004, Science, 303, 672-676; Pal-Bhadra et al., 2004, Science, 303, 669-672; Allshire, 2002, Science, 297, 1818-1819; Volpe et al., 2002, Science, 297, 1833-1837; Jenuwein, 2002, Science, 297, 2215-2218; and Hall et al., 2002, Science, 297, 2232-2237). In another non-limiting example, modulation of gene expression by siNA molecules of the invention can result from siNA mediated cleavage of RNA (either coding or non-coding RNA) via RISC, or alternately, translational inhibition as is known in the art. In another embodiment, modulation of gene expression by siNA molecules of the invention can result from transcriptional inhibition (see for example Janowski et al., 2005, Nature Chemical Biology, 1, 216-222).

[0321] By "asymmetric hairpin" as used herein is meant a linear siNA molecule comprising an antisense region, a loop

portion that can comprise nucleotides or non-nucleotides, and a sense region that comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complementary nucleotides to base pair with the antisense region and form a duplex with loop. For example, an asymmetric hairpin siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (e.g. about 15 to about 30, or about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides) and a loop region comprising about 4 to about 12 (e.g., about 4, 5, 6, 7, 8, 9, 10, 11, or 12) nucleotides, and a sense region having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides that are complementary to the antisense region. The asymmetric hairpin siNA molecule can also comprise a 5'-terminal phosphate group that can be chemically modified. The loop portion of the asymmetric hairpin siNA molecule can comprise nucleotides, non-nucleotides, linker molecules, or conjugate molecules as described herein.

[0322] By "asymmetric duplex" as used herein is meant a siNA molecule having two separate strands comprising a sense region and an antisense region, wherein the sense region comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complementary nucleotides to base pair with the antisense region and form a duplex. For example, an asymmetric duplex siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (e.g. about 15 to about 30, or about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides) and a sense region having about 3 to about 25 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) nucleotides that are complementary to the antisense region.

[0323] The term "polynucleotide" or "nucleic acid molecule" as used herein, refers to a molecule having nucleotides. The nucleic acid can be single, double, or multiple stranded and can comprise modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof.

[0324] The term "enzymatic nucleic acid molecule" as used herein refers to a nucleic acid molecule which has complementarity in a substrate binding region to a specified gene target, and also has an enzymatic activity which is active to specifically cleave target RNA. That is, the enzymatic nucleic acid molecule is able to intermolecularly cleave RNA and thereby inactivate a target RNA molecule. These complementary regions allow sufficient hybridization of the enzymatic nucleic acid molecule to the target RNA and thus permit cleavage. One hundred percent complementarity is preferred, but complementarity as low as 50-75% can also be useful in this invention (see for example Werner and Uhlenbeck, 1995, Nucleic Acids Research, 23, 2092-2096; Hammann et al., 1999, Antisense and Nucleic Acid Drug Dev., 9, 25-31). The nucleic acids can be modified at the base, sugar, and/or phosphate groups. The term enzymatic nucleic acid is used interchangeably with phrases such as ribozymes, catalytic RNA, enzymatic RNA, catalytic DNA, aptazyme or aptamer-binding ribozyme, regulatable ribozyme, catalytic oligonucleotides, nucleozyme, DNAzyme, RNA enzyme, endoribonuclease, endonuclease, minizyme, leadzyme, oligozyme or DNA enzyme. All of

these terminologies describe nucleic acid molecules with enzymatic activity. The specific enzymatic nucleic acid molecules described in the instant application are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target nucleic acid regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart a nucleic acid cleaving and/or ligation activity to the molecule (Cech et al., U.S. Pat. No. 4,987,071; Cech et al., 1988, 260 JAMA 3030). Ribozymes and enzymatic nucleic molecules of the invention can be chemically modified as is generally known in the art or as described herein.

[0325] The term "antisense nucleic acid", as used herein, refers to a non-enzymatic nucleic acid molecule that binds to target RNA by means of RNA-RNA or RNA-DNA or RNA-PNA (protein nucleic acid; Egholm et al., 1993 Nature 365, 566) interactions and alters the activity of the target RNA (for a review, see Stein and Cheng, 1993 Science 261, 1004 and Woolf et al., U.S. Pat. No. 5,849,902). Typically, antisense molecules are complementary to a target sequence along a single contiguous sequence of the antisense molecule. However, in certain embodiments, an antisense molecule can bind to substrate such that the substrate molecule forms a loop, and/or an antisense molecule can bind such that the antisense molecule forms a loop. Thus, the antisense molecule can be complementary to two (or even more) non-contiguous substrate sequences or two (or even more) non-contiguous sequence portions of an antisense molecule can be complementary to a target sequence or both. For a review of current antisense strategies, see Schmajuk et al., 1999, J. Biol. Chem., 274, 21783-21789, Delihas et al., 1997, Nature, 15, 751-753, Stein et al., 1997, Antisense N. A. Drug Dev., 7, 151, Crooke, 2000, Methods Enzymol., 313, 3-45; Crooke, 1998, Biotech. Genet. Eng. Rev., 15, 121-157, Crooke, 1997, Ad. Pharmacol., 40, 1-49. In addition, antisense DNA can be used to target RNA by means of DNA-RNA interactions, thereby activating RNase H, which digests the target RNA in the duplex. The antisense oligonucleotides can comprise one or more RNAse H activating region, which is capable of activating RNAse H cleavage of a target RNA. Antisense DNA can be synthesized chemically or expressed via the use of a single stranded DNA expression vector or equivalent thereof. Antisense molecules of the invention can be chemically modified as is generally known in the art or as described herein.

[0326] The term "RNase H activating region" as used herein, refers to a region (generally greater than or equal to 4-25 nucleotides in length, preferably from 5-11 nucleotides in length) of a nucleic acid molecule capable of binding to a target RNA to form a non-covalent complex that is recognized by cellular RNase H enzyme (see for example Arrow et al., U.S. Pat. No. 5,849,902; Arrow et al., U.S. Pat. No. 5,989,912). The RNase H enzyme binds to the nucleic acid molecule-target RNA complex and cleaves the target RNA sequence. The RNase H activating region comprises, for example, phosphodiester, phosphorothioate (preferably at least four of the nucleotides are phosphorothioate substitutions; more specifically, 4-11 of the nucleotides are phosphorothiote substitutions); phosphorodithioate, 5'-thiophosphate, or methylphosphonate backbone chemistry or a combination thereof. In addition to one or more backbone

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chemistries described above, the RNase H activating region can also comprise a variety of sugar chemistries. For example, the RNase H activating region can comprise deoxyribose, arabino, fluoroarabino or a combination thereof, nucleotide sugar chemistry. Those skilled in the art will recognize that the foregoing are non-limiting examples and that any combination of phosphate, sugar and base chemistry of a nucleic acid that supports the activity of RNase H enzyme is within the scope of the definition of the RNase H activating region and the instant invention.

[0327] The term "2-5A antisense chimera" as used herein, refers to an antisense oligonucleotide containing a 5'-phosphorylated 2'-5'-linked adenylate residue. These chimeras bind to target RNA in a sequence-specific manner and activate a cellular 2-5A-dependent ribonuclease which, in turn, cleaves the target RNA (Torrence et al., 1993 *Proc. Natl. Acad. Sci. USA* 90, 1300; Silverman et al., 2000, Methods Enzymol., 313, 522-533; Player and Torrence, 1998, Pharmacol. Ther., 78, 55-113). 2-5A antisense chimera molecules of the invention can be chemically modified as is generally known in the art or as described herein.

[0328] The term "triplex forming oligonucleotides" as used herein, refers to an oligonucleotide that can bind to a double-stranded DNA in a sequence-specific manner to form a triple-strand helix. Formation of such triple helix structure has been shown to inhibit transcription of the targeted gene (Duval-Valentin et al., 1992 Proc. Natl. Acad. Sci. USA 89, 504; Fox, 2000, Curr. Med. Chem., 7, 17-37; Praseuth et. al., 2000, Biochim. Biophys. Acta, 1489, 181-206). Triplex forming oligonucleotide molecules of the invention can be chemically modified as is generally known in the art or as described herein.

[0329] The term "decoy RNA" as used herein, refers to a RNA molecule or aptamer that is designed to preferentially bind to a predetermined ligand. Such binding can result in the inhibition or activation of a target molecule. The decoy RNA or aptamer can compete with a naturally occurring binding target for the binding of a specific ligand. For example, it has been shown that over-expression of HIV trans-activation response (TAR) RNA can act as a "decoy" and efficiently binds HIV tat protein, thereby preventing it from binding to TAR sequences encoded in the HIV RNA (Sullenger et al., 1990, Cell, 63, 601-608). This is but a specific example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art, see for example Gold et al., 1995, Annu. Rev. Biochem., 64, 763; Brody and Gold, 2000, J. Biotechnol., 74, 5; Sun, 2000, Curr. Opin. Mol. Ther., 2, 100; Kusser, 2000, J. Biotechnol., 74, 27; Hermann and Patel, 2000, Science, 287, 820; and Jayasena, 1999, Clinical Chemistry, 45, 1628. Similarly, a decoy RNA can be designed to bind to a receptor and block the binding of an effector molecule or a decoy RNA can be designed to bind to receptor of interest and prevent interaction with the receptor. Decoy molecules of the invention can be chemically modified as is generally known in the art or as described herein.

[0330] The term "single stranded RNA" (ssRNA) as used herein refers to a naturally occurring or synthetic ribonucleic acid molecule comprising a linear single strand, for example a ssRNA can be a messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA) etc. of a gene.

[0331] The term "single stranded DNA" (ssDNA) as used herein refers to a naturally occurring or synthetic deoxyribonucleic acid molecule comprising a linear single strand, for example, a ssDNA can be a sense or antisense gene sequence or EST (Expressed Sequence Tag).

[0332] The term "double stranded RNA" or "dsRNA" as used herein refers to a double stranded RNA molecule capable of RNA interference, including short interfering RNA (siNA).

[0333] The term "allozyme" as used herein refers to an allosteric enzymatic nucleic acid molecule, see for example see for example George et al., U.S. Pat. Nos. 5,834,186 and 5,741,679, Shih et al., U.S. Pat. No. 5,589,332, Nathan et al., U.S. Pat. No. 5,871,914, Nathan and Ellington, International PCT publication No. WO 00/24931, Breaker et al., International PCT Publication Nos. WO 00/26226 and 98/27104, and Sullenger et al., International PCT publication No. WO 99/29842.

[0334] By "aptamer" or "nucleic acid aptamer" as used herein is meant a polynucleotide that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that is distinct from sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art, see for example Gold et al., 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science*, 287, 820; and Jayasena, 1999, *Clinical Chemistry*, 45, 1628. Aptamer molecules of the invention can be chemically modified as is generally known in the art or as described herein.

[0335] By "modulate" is meant that the expression of the gene, or level of RNA molecule or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

[0336] By "inhibit", "down-regulate", or "reduce", it is meant that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is reduced below that observed in the absence of the nucleic acid molecules (e.g., siNA) of the invention. In one embodiment, inhibition, down-regulation or reduction with a siNA molecule is below that level observed in the presence of an inactive or attenuated molecule. In another embodiment, inhibition, down-regulation, or reduction with siNA molecules is below that level observed in the presence of, for example, a siNA molecule with scrambled sequence or with mismatches. In another embodiment, inhibition, down-regulation, or reduction of

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gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence. In one embodiment, inhibition, down regulation, or reduction of gene expression is associated with post transcriptional silencing, such as RNAi mediated cleavage of a target nucleic acid molecule (e.g. RNA) or inhibition of translation. In one embodiment, inhibition, down regulation, or reduction of gene expression is associated with pretranscriptional silencing.

[0337] By "up-regulate", or "promote", it is meant that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is increased above that observed in the absence of the nucleic acid molecules (e.g., siNA) of the invention. In one embodiment, up-regulation or promotion of gene expression with an siNA molecule is above that level observed in the presence of an inactive or attenuated molecule. In another embodiment, up-regulation or promotion of gene expression with siNA molecules is above that level observed in the presence of, for example, an siNA molecule with scrambled sequence or with mismatches. In another embodiment, up-regulation or promotion of gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence. In one embodiment, up-regulation or promotion of gene expression is associated with inhibition of RNA mediated gene silencing, such as RNAi mediated cleavage or silencing of a coding or non-coding RNA target that down regulates, inhibits, or silences the expression of the gene of interest to be up-regulated. The down regulation of gene expression can, for example, be induced by a coding RNA or its encoded protein, such as through negative feedback or antagonistic effects. The down regulation of gene expression can, for example, be induced by a non-coding RNA having regulatory control over a gene of interest, for example by silencing expression of the gene via translational inhibition, chromatin structure, methylation, RISC mediated RNA cleavage, or translational inhibition. As such, inhibition or down regulation of targets that down regulate, suppress, or silence a gene of interest can be used to up-regulate or promote expression of the gene of interest toward therapeutic use.

[0338] By "gene", or "target gene", is meant a nucleic acid that encodes RNA, for example, nucleic acid sequences including, but not limited to, structural genes encoding a polypeptide. A gene or target gene can also encode a functional RNA (fRNA) or non-coding RNA (ncRNA), such as small temporal RNA (stRNA), micro RNA (miRNA), small nuclear RNA (snRNA), short interfering RNA (siRNA), small nucleolar RNA (snRNA), ribosomal RNA (rRNA), transfer RNA (tRNA) and precursor RNAs thereof. Such non-coding RNAs can serve as target nucleic acid molecules for siNA mediated RNA interference in modulating the activity of fRNA or ncRNA involved in functional or regulatory cellular processes. Abberant fRNA or ncRNA activity leading to disease can therefore be modulated by siNA molecules of the invention. siNA molecules targeting fRNA and ncRNA can also be used to manipulate or alter the genotype or phenotype of a subject, organism or cell, by intervening in cellular processes such as genetic imprinting, transcription, translation, or nucleic acid processing (e.g., transamination, methylation etc.). The target gene can be a gene derived from a cell, an endogenous gene, a transgene,

or exogenous genes such as genes of a pathogen, for example a virus, which is present in the cell after infection thereof. The cell containing the target gene can be derived from or contained in any organism, for example a plant, animal, protozoan, virus, bacterium, or fungus. Non-limiting examples of plants include monocots, dicots, or gymnosperms. Non-limiting examples of animals include vertebrates or invertebrates. Non-limiting examples of fungi include molds or yeasts. For a review, see for example Snyder and Gerstein, 2003, *Science*, 300, 258-260.

[0339] By "target" as used herein is meant, any target protein, peptide, or polypeptide encoded by a target gene. The term "target" also refers to nucleic acid sequences encoding any target protein, peptide, or polypeptide having target activity, such as encoded by target RNA. The term "target" is also meant to include other target encoding sequence, such as other target isoforms, mutant target genes, splice variants of target genes, and target gene polymorphisms. By "target nucleic acid" is meant any nucleic acid sequence whose expression or activity is to be modulated. The target nucleic acid can be DNA or RNA.

[0340] By "non-canonical base pair" is meant any non-Watson Crick base pair, such as mismatches and/or wobble base pairs, including flipped mismatches, single hydrogen bond mismatches, trans-type mismatches, triple base interactions, and quadruple base interactions. Non-limiting examples of such non-canonical base pairs include, but are not limited to, AC reverse Hoogsteen, AC wobble, AU reverse Hoogsteen, GU wobble, AA N7 amino, CC 2-carbonyl-amino(H1)-N-3-amino(H2), GA sheared, UC 4-carbonyl-amino, UU imino-carbonyl, AC reverse wobble, AU Hoogsteen, AU reverse Watson Crick, CG reverse Watson Crick, GC N3-amino-amino N3, AA Ni-amino symmetric, AA N7-amino symmetric, GA N7-N1 amino-carbonyl, GA+ carbonyl-amino N7-N1, GG N1-carbonyl symmetric, GG N3-amino symmetric, CC carbonyl-amino symmetric, CC N3-amino symmetric, UU 2-carbonyl-imino symmetric, UU 4-carbonyl-imino symmetric, AA amino-N3, AA N1-amino, AC amino 2-carbonyl, AC N3-amino, AC N7-amino, AU amino-4-carbonyl, AU N1-imino, AU N3-imino, AU N7-imino, CC carbonyl-amino, GA amino-N1, GA amino-N7, GA carbonyl-amino, GA N3-amino, GC amino-N3, GC carbonyl-amino, GC N3-amino, GC N7-amino, GG amino-N7, GG carbonyl-imino, GG N7-amino, GU amino-2-carbonyl, GU carbonyl-imino, GU imino-2-carbonyl, GU N7-imino, psiU imino-2-carbonyl, UC 4-carbonyl-amino, UC imino-carbonyl, UU imino-4-carbonyl, AC C2-H-N3, GA carbonyl-C2-H, UU imino-4-carbonyl 2 carbonyl-C5-H, AC amino(A) N3(C)-carbonyl, GC imino amino-carbonyl, Gpsi imino-2-carbonyl amino-2-carbonyl, and GU imino amino-2-carbonyl base pairs.

[0341] By "target" as used herein is meant, any target protein, peptide, or polypeptide, such as encoded by Genbank Accession Nos. shown in U.S. Ser. No. 10/923,536 and U.S. Ser. No. 10/923,536, both incorporated by reference herein. The term "target" also refers to nucleic acid sequences or target polynucleotide sequence encoding any target protein, peptide, or polypeptide, such as proteins, peptides, or polypeptides encoded by sequences having Genbank Accession Nos. shown in U.S. Ser. No. 10/923,536 and U.S. Ser. No. 10/923,536. The target of interest can include target polynucleotide sequences, such as target DNA or target RNA. The term "target" is also meant to include

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other sequences, such as differing isoforms, mutant target genes, splice variants of target polynucleotides, target polymorphisms, and non-coding (e.g., ncRNA, miRNA, sRNA) or other regulatory polynucleotide sequences as described herein. Therefore, in various embodiments of the invention, a double stranded nucleic acid molecule of the invention (e.g., siNA) having complementarity to a target RNA can be used to inhibit or down regulate miRNA or other ncRNA activity. In one embodiment, inhibition of miRNA or ncRNA activity can be used to down regulate or inhibit gene expression (e.g., gene targets described herein or otherwise known in the art) or viral replication (e.g., viral targets described herein or otherwise known in the art) that is dependent on miRNA or ncRNA activity. In another embodiment, inhibition of miRNA or ncRNA activity by double stranded nucleic acid molecules of the invention (e.g. siNA) having complementarity to the miRNA or ncRNA can be used to up regulate or promote target gene expression (e.g., gene targets described herein or otherwise known in the art) where the expression of such genes is down regulated, suppressed, or silenced by the miRNA or ncRNA. Such up-regulation of gene expression can be used to treat diseases and conditions associated with a loss of function or haploinsufficiency as are generally known in the art (e.g., muscular dystrophies, cystic fibrosis, or neurologic diseases and conditions described herein such as epilepsy, including severe myoclonic epilepsy of infancy or Dravet syndrome).

[0342] By "homologous sequence" is meant, a nucleotide sequence that is shared by one or more polynucleotide sequences, such as genes, gene transcripts and/or non-coding polynucleotides. For example, a homologous sequence can be a nucleotide sequence that is shared by two or more genes encoding related but different proteins, such as different members of a gene family, different protein epitopes, different protein isoforms or completely divergent genes, such as a cytokine and its corresponding receptors. A homologous sequence can be a nucleotide sequence that is shared by two or more non-coding polynucleotides, such as noncoding DNA or RNA, regulatory sequences, introns, and sites of transcriptional control or regulation. Homologous sequences can also include conserved sequence regions shared by more than one polynucleotide sequence. Homology does not need to be perfect homology (e.g., 100%), as partially homologous sequences are also contemplated by the instant invention (e.g., 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80% etc.).

[0343] By "conserved sequence region" is meant, a nucleotide sequence of one or more regions in a polynucleotide does not vary significantly between generations or from one biological system, subject, or organism to another biological system, subject, or organism. The polynucleotide can include both coding and non-coding DNA and RNA.

[0344] By "sense region" is meant a nucleotide sequence of a siNA molecule having complementarity to an antisense region of the siNA molecule. In addition, the sense region of a siNA molecule can comprise a nucleic acid sequence having homology with a target nucleic acid sequence. In one embodiment, the sense region of the siNA molecule is referred to as the sense strand or passenger strand.

[0345] By "antisense region" is meant a nucleotide sequence of a siNA molecule having complementarity to a target nucleic acid sequence. In addition, the antisense region of a siNA molecule can optionally comprise a nucleic acid sequence having complementarity to a sense region of the siNA molecule. In one embodiment, the antisense region of the siNA molecule is referred to as the antisense strand or guide strand.

[0346] By "target nucleic acid" or "target polynucleotide" is meant any nucleic acid sequence whose expression or activity is to be modulated. The target nucleic acid can be DNA or RNA. In one embodiment, a target nucleic acid of the invention is target RNA or DNA.

[0347] By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types as described herein. In one embodiment, a double stranded nucleic acid molecule of the invention, such as an siNA molecule, wherein each strand is between 15 and 30 nucleotides in length, comprises between about 10% and about 100% (e.g., about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%) complementarity between the two strands of the double stranded nucleic acid molecule. In another embodiment, a double stranded nucleic acid molecule of the invention, such as an siNA molecule, where one strand is the sense strand and the other stand is the antisense strand, wherein each strand is between 15 and 30 nucleotides in length, comprises between at least about 10% and about 100% (e.g., at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%) complementarity between the nucleotide sequence in the antisense strand of the double stranded nucleic acid molecule and the nucleotide sequence of its corresponding target nucleic acid molecule, such as a target RNA or target mRNA or viral RNA. In one embodiment, a double stranded nucleic acid molecule of the invention, such as an siNA molecule, where one strand comprises nucleotide sequence that is referred to as the sense region and the other strand comprises a nucleotide sequence that is referred to as the antisense region, wherein each strand is between 15 and 30 nucleotides in length, comprises between about 10% and about 100% (e.g., about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%) complementarity between the sense region and the antisense region of the double stranded nucleic acid molecule. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner et al., 1987, *CSH Symp. Quant. Biol.* LII pp. 123-133; Frier et al., 1986, *Proc. Nat. Acad. Sci. USA* 83:9373-9377; Turner et al., 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, or 10 nucleotides out of a total of 10 nucleotides in the first oligonucleotide being based paired to a second nucleic acid sequence having 10 nucleotides represents 50%, 60%, 70%, 80%, 90%, and 100% complementary respectively). In one embodiment, a siNA molecule of the invention has perfect complementarity between the sense strand or sense region and the antisense strand or antisense region of the siNA molecule. In one embodiment, a siNA molecule of the invention is perfectly complementary to a corresponding target nucleic acid mol-

ecule. "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. In one embodiment, a siNA molecule of the invention comprises about 15 to about 30 or more (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 or more) nucleotides that are complementary to one or more target nucleic acid molecules or a portion thereof. In one embodiment, a siNA molecule of the invention has partial complementarity (i.e., less than 100% complementarity) between the sense strand or sense region and the antisense strand or antisense region of the siNA molecule or between the antisense strand or antisense region of the siNA molecule and a corresponding target nucleic acid molecule. For example, partial complementarity can include various mismatches or non-based paired nucleotides (e.g., 1, 2, 3, 4, 5 or more mismatches or non-based paired nucleotides) within the siNA structure which can result in bulges, loops, or overhangs that result between the between the sense strand or sense region and the antisense strand or antisense region of the siNA molecule or between the antisense strand or antisense region of the siNA molecule and a corresponding target nucleic acid molecule.

[0348] In one embodiment, a double stranded nucleic acid molecule of the invention, such as siNA molecule, has perfect complementarity between the sense strand or sense region and the antisense strand or antisense region of the nucleic acid molecule. In one embodiment, double stranded nucleic acid molecule of the invention, such as siNA molecule, is perfectly complementary to a corresponding target nucleic acid molecule.

[0349] In one embodiment, double stranded nucleic acid molecule of the invention, such as siNA molecule, has partial complementarity (i.e., less than 100% complementarity) between the sense strand or sense region and the antisense strand or antisense region of the double stranded nucleic acid molecule or between the antisense strand or antisense region of the nucleic acid molecule and a corresponding target nucleic acid molecule. For example, partial complementarity can include various mismatches or non-base paired nucleotides (e.g., 1, 2, 3, 4, 5 or more mismatches or non-based paired nucleotides, such as nucleotide bulges) within the double stranded nucleic acid molecule, structure which can result in bulges, loops, or overhangs that result between the sense strand or sense region and the antisense strand or antisense region of the double stranded nucleic acid molecule or between the antisense strand or antisense region of the double stranded nucleic acid molecule and a corresponding target nucleic acid molecule.

[0350] In one embodiment, double stranded nucleic acid molecule of the invention is a microRNA (miRNA). By "mircoRNA" or "miRNA" is meant, a small double stranded RNA that regulates the expression of target messenger RNAs either by mRNA cleavage, translational repression/inhibition or heterochromatic silencing (see for example Ambros, 2004, Nature, 431, 350-355; Bartel, 2004, Cell, 116, 281-297; Cullen, 2004, Virus Research., 102, 3-9; He et al., 2004, Nat. Rev. Genet., 5, 522-531; and Ying et al., 2004, Gene, 342, 25-28). In one embodiment, the microRNA of the invention, has partial complementarity (i.e., less than 100% complementarity) between the sense strand or sense region and the antisense strand or antisense region of the miRNA molecule or between the antisense strand or anti-

sense region of the miRNA and a corresponding target nucleic acid molecule. For example, partial complementarity can include various mismatches or non-base paired nucleotides (e.g., 1, 2, 3, 4, 5 or more mismatches or non-based paired nucleotides, such as nucleotide bulges) within the double stranded nucleic acid molecule, structure which can result in bulges, loops, or overhangs that result between the sense strand or sense region and the antisense strand or antisense region of the miRNA or between the antisense strand or antisense region of the miRNA and a corresponding target nucleic acid molecule.

[0351] In one embodiment, compositions of the invention such as formulated molecular compositions and formulated siNA compositions of the invention that down regulate or reduce target gene expression are used for preventing or treating diseases, disorders, conditions, or traits in a subject or organism as described herein or otherwise known in the art.

[0352] By "proliferative disease" or "cancer" as used herein is meant, any disease, condition, trait, genotype or phenotype characterized by unregulated cell growth or replication as is known in the art; including leukemias, for example, acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), and chronic lymphocytic leukemia, AIDS related cancers such as Kaposi's sarcoma; breast cancers; bone cancers such as Osteosarcoma, Chondrosarcomas, Ewing's sarcoma, Fibrosarcomas, Giant cell tumors, Adamantinomas, and Chordomas; Brain cancers such as Meningiomas, Glioblastomas, Lower-Grade Astrocytomas, Oligodendrocytomas, Pituitary Tumors, Schwannomas, and Metastatic brain cancers; cancers of the head and neck including various lymphomas such as mantle cell lymphoma, non-Hodgkins lymphoma, adenoma, squamous cell carcinoma, laryngeal carcinoma, gallbladder and bile duct cancers, cancers of the retina such as retinoblastoma, cancers of the esophagus, gastric cancers, multiple myeloma, ovarian cancer, uterine cancer, thyroid cancer, testicular cancer, endometrial cancer, melanoma, colorectal cancer, lung cancer, bladder cancer, prostate cancer, lung cancer (including non-small cell lung carcinoma), pancreatic cancer, sarcomas, Wilms' tumor, cervical cancer, head and neck cancer, skin cancers, nasopharyngeal carcinoma, liposarcoma, epithelial carcinoma, renal cell carcinoma, gallbladder adeno carcinoma, parotid adenocarcinoma, endometrial sarcoma, multidrug resistant cancers; and proliferative diseases and conditions, such as neovascularization associated with tumor angiogenesis, macular degeneration (e.g., wet/dry AMD), corneal neovascularization, diabetic retinopathy, neovascular glaucoma, myopic degeneration and other proliferative diseases and conditions such as restenosis and polycystic kidney disease, and any other cancer or proliferative disease, condition, trait, genotype or phenotype that can respond to the modulation of disease related gene expression in a cell or tissue, alone or in combination with other therapies.

[0353] By "inflammatory disease" or "inflammatory condition" as used herein is meant any disease, condition, trait, genotype or phenotype characterized by an inflammatory or allergic process as is known in the art, such as inflammation, acute inflammation, chronic inflammation, respiratory disease, atherosclerosis, psoriasis, dermatitis, restenosis, asthma, allergic rhinitis, atopic dermatitis, septic shock,

rheumatoid arthritis, inflammatory bowl disease, inflammatory pelvic disease, pain, ocular inflammatory disease, celiac disease, Leigh Syndrome, Glycerol Kinase Deficiency, Familial eosinophilia (FE), autosomal recessive spastic ataxia, laryngeal inflammatory disease; Tuberculosis, Chronic cholecystitis, Bronchiectasis, Silicosis and other pneumoconioses, and any other inflammatory disease, condition, trait, genotype or phenotype that can respond to the modulation of disease related gene expression in a cell or tissue, alone or in combination with other therapies.

[0354] By "autoimmune disease" or "autoimmune condition" as used herein is meant, any disease, condition, trait, genotype or phenotype characterized by autoimmunity as is known in the art, such as multiple sclerosis, diabetes mellitus, lupus, celiac disease, Crohn's disease, ulcerative colitis, Guillain-Barre syndrome, scleroderms, Goodpasture's syndrome, Wegener's granulomatosis, autoimmune epilepsy, Rasmussen's encephalitis, Primary biliary sclerosis, Sclerosing cholangitis, Autoimmune hepatitis, Addison's disease, Hashimoto's thyroiditis, Fibromyalgia, Menier's syndrome; transplantation rejection (e.g., prevention of allograft rejection) pernicious anemia, rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, Sjogren's syndrome, lupus erythematosus, multiple sclerosis, myasthenia gravis, Reiter's syndrome, Grave's disease, and any other autoimmune disease, condition, trait, genotype or phenotype that can respond to the modulation of disease related gene expression in a cell or tissue, alone or in combination with other therapies.

[0355] By "infectious disease" is meant any disease, condition, trait, genotype or phenotype associated with an infectious agent, such as a virus, bacteria, fungus, prion, or parasite. Non-limiting examples of various viral genes that can be targeted using siNA molecules of the invention include Hepatitis C Virus (HCV, for example Genbank Accession Nos: D11168, D50483.1, L38318 and S82227), Hepatitis B Virus (HBV, for example GenBank Accession No. AF100308.1), Human Immunodeficiency Virus type 1 (HIV-1, for example GenBank Accession No. U51188), Human Immunodeficiency Virus type 2 (HIV-2, for example GenBank Accession No. X60667), West Nile Virus (WNV for example GenBank accession No. NC_001563), cytomegalovirus (CMV for example GenBank Accession No. NC_001347), respiratory syncytial virus (RSV for example GenBank Accession No. NC_001781), influenza virus (for example GenBank Accession No. AF037412, rhinovirus (for example, GenBank accession numbers: D00239, X02316, X01087, L24917, M16248, K02121, X01087), papillomavirus (for example GenBank Accession No. NC_001353), Herpes Simplex Virus (HSV for example GenBank Accession No. NC_001345), and other viruses such as HTLV (for example GenBank Accession No. AJ430458). Due to the high sequence variability of many viral genomes, selection of siNA molecules for broad therapeutic applications would likely involve the conserved regions of the viral genome. Nonlimiting examples of conserved regions of the viral genomes include but are not limited to 5'-Non Coding Regions (NCR), 3'-Non Coding Regions (NCR) and/or internal ribosome entry sites (IRES). siNA molecules designed against conserved regions of various viral genomes will enable efficient inhibition of viral replication in diverse patient populations and may ensure the effectiveness of the siNA molecules against viral quasi species which evolve due to mutations in the non-conserved regions of the viral

genome. Non-limiting examples of bacterial infections include Actinomycosis, Anthrax, Aspergillosis, Bacteremia, Bacterial Infections and Mycoses, Bartonella Infections, Botulism, Brucellosis, Burkholderia Infections, Campylobacter Infections, Candidiasis, Cat-Scratch Disease, Chlamydia Infections, Cholera, Clostridium Infections, Coccidioidomycosis, Cross Infection, Cryptococcosis, Dermatomycoses, Dermatomycoses, Diphtheria, Ehrlichiosis, Escherichia coli Infections, Fasciitis, Necrotizing, Fusobacterium Infections, Gas Gangrene, Gram-Negative Bacterial Infections, Gram-Positive Bacterial Infections, Histoplasmosis, Impetigo, Klebsiella Infections, Legionellosis, Leprosy, Leptospirosis, Listeria Infections, Lyme Disease, Maduromycosis, Melioidosis, Mycobacterium Infections, Mycoplasma Infections, Mycoses, Nocardia Infections, Onychomycosis, Ornithosis, Plague, Pneumococcal Infections, Pseudomonas Infections, Q Fever, Rat-Bite Fever, Relapsing Fever, Rheumatic Fever, Rickettsia Infections, Rocky Mountain Spotted Fever, Salmonella Infections, Scarlet Fever, Scrub Typhus, Sepsis, Sexually Transmitted Diseases—Bacterial, Bacterial Skin Diseases, Staphylococcal Infections, Streptococcal Infections, Tetanus, Tick-Borne Diseases, Tuberculosis, Tularemia, Typhoid Fever, Typhus, Epidemic Louse-Borne, Vibrio Infections, Yaws, Yersinia Infections, Zoonoses, and Zygomycosis. Non-limiting examples of fungal infections include Aspergillosis, Blastomycosis, Coccidioidomycosis, Cryptococcosis, Fungal Infections of Fingernails and Toenails, Fungal Sinusitis, Histoplasmosis, Histoplasmosis, Mucormycosis, Nail Fungal Infection, Paracoccidioidomycosis, Sporotrichosis, Valley Fever (Coccidioidomycosis), and Mold Allergy.

[0356] By "neurologic disease" or "neurological disease" is meant any disease, disorder, or condition affecting the central or peripheral nervous system, inlcuding ADHD, AIDS—Neurological Complications, Absence of the Septum Pellucidum, Acquired Epileptiform Aphasia, Acute Disseminated Encephalomyelitis, Adrenoleukodystrophy, Agenesis of the Corpus Callosum, Agnosia, Aicardi Syndrome, Alexander Disease, Alpers' Disease, Alternating Hemiplegia, Alzheimer's Disease, Amyotrophic Lateral Sclerosis, Anencephaly, Aneurysm, Angelman Syndrome, Angiomatosis, Anoxia, Aphasia, Apraxia, Arachnoid Cysts, Arachnoiditis, Arnold-Chiari Malformation, Arteriovenous Malformation, Aspartame, Asperger Syndrome, Ataxia Telangiectasia, Ataxia, Attention Deficit-Hyperactivity Disorder, Autism, Autonomic Dysfunction, Back Pain, Barth Syndrome, Batten Disease, Behcet's Disease, Bell's Palsy, Benign Essential Blepharospasm, Benign Focal Amyotrophy, Benign Intracranial Hypertension, Bernhardt-Roth Syndrome, Binswanger's Disease, Blepharospasm, Bloch-Sulzberger Syndrome, Brachial Plexus Birth Injuries, Brachial Plexus Injuries, Bradbury-Eggleston Syndrome, Brain Aneurysm, Brain Injury, Brain and Spinal Tumors, Brown-Sequard Syndrome, Bulbospinal Muscular Atrophy, Canavan Disease, Carpal Tunnel Syndrome, Causalgia, Cavernomas, Cavernous Angioma, Cavernous Malformation, Central Cervical Cord Syndrome, Central Cord Syndrome, Central Pain Syndrome, Cephalic Disorders, Cerebellar Degeneration, Cerebellar Hypoplasia, Cerebral Aneurysm, Cerebral Arteriosclerosis, Cerebral Atrophy, Cerebral Beriberi, Cerebral Gigantism, Cerebral Hypoxia, Cerebral Palsy, Cerebro-Oculo-Facio-Skeletal Syndrome, Charcot-Marie-Tooth Disorder, Chiari Malformation, Chorea, Choreoacanthocytosis, Chronic Inflammatory Demyelinating Polyneur-

opathy (CIDP), Chronic Orthostatic Intolerance, Chronic Pain, Cockayne Syndrome Type II, Coffin Lowry Syndrome, Coma, including Persistent Vegetative State, Complex Regional Pain Syndrome, Congenital Facial Diplegia, Congenital Myasthenia, Congenital Myopathy, Congenital Vascular Cavernous Malformations, Corticobasal Degeneration, Cranial Arteritis, Craniosynostosis, Creutzfeldt-Jakob Disease, Cumulative Trauma Disorders, Cushing's Syndrome, Cytomegalic Inclusion Body Disease (CIBD), Cytomegalovirus Infection, Dancing Eyes-Dancing Feet Syndrome, Dandy-Walker Syndrome, Dawson Disease, De Morsier's Syndrome, Dejerine-Klumpke Palsy, Dementia—Multi-Infarct, Dementia—Subcortical, Dementia With Lewy Bodies, Dermatomyositis, Developmental Dyspraxia, Devic's Syndrome, Diabetic Neuropathy, Diffuse Sclerosis, Dravet's Syndrome; Dysautonomia, Dysgraphia, Dyslexia, Dysphagia, Dyspraxia, Dystonias, Early Infantile Epileptic Encephalopathy, Empty Sella Syndrome, Encephalitis Lethargica, Encephalitis and Meningitis, Encephaloceles, Encephalopathy, Encephalotrigeminal Angiomatosis, Epilepsy, Erb's Palsy, Erb-Duchenne and Dejerine-Klumpke Palsies, Fabry's Disease, Fahr's Syndrome, Fainting, Familial Dysautonomia, Familial Hemangioma, Familial Idiopathic Basal Ganglia Calcification, Familial Spastic Paralysis, Febrile Seizures (e.g., GEFS and GEFS plus), Fisher Syndrome, Floppy Infant Syndrome, Friedreich's Ataxia, Gaucher's Disease, Gerstmann's Syndrome, Gerstmann-Straussler-Scheinker Disease, Giant Cell Arteritis, Giant Cell Inclusion Disease, Globoid Cell Leukodystrophy, Glossopharyngeal Neuralgia, Guillain-Barre Syndrome, HTLV-1 Associated Myelopathy, Hallervorden-Spatz Disease, Head Injury, Headache, Hemicrania Continua, Hemifacial Spasm, Hemiplegia Alterans, Hereditary Neuropathies, Hereditary Spastic Paraplegia, Heredopathia Atactica Polyneuritiformis, Herpes Zoster Oticus, Herpes Zoster, Hirayama Syndrome, Holoprosencephaly, Huntington's Disease, Hydranencephaly, Hydrocephalus—Normal Pressure, Hydrocephalus, Hydromyelia, Hypercortisolism, Hypersomnia, Hypertonia, Hypotonia, Hypoxia, Immune-Mediated Encephalomyelitis, Inclusion Body Myositis, Incontinentia Pigmenti, Infantile Hypotonia, Infantile Phytanic Acid Storage Disease, Infantile Refsum Disease, Infantile Spasms, Inflammatory Myopathy, Intestinal Lipodystrophy, Intracranial Cysts, Intracranial Hypertension, Isaac's Syndrome, Joubert Syndrome, Kearns-Sayre Syndrome, Kennedy's Disease, Kinsbourne syndrome, Kleine-Levin syndrome, Klippel Feil Syndrome, Klippel-Trenaunay Syndrome (KTS), Klüver-Bucy Syndrome, Korsakoffs Amnesic Syndrome, Krabbe Disease, Kugelberg-Welander Disease, Kuru, Lambert-Eaton Myasthenic Syndrome, Landau-Kleffner Syndrome, Lateral Femoral Cutaneous Nerve Entrapment, Lateral Medullary Syndrome, Learning Disabilities, Leigh's Disease, Lennox-Gastaut Syndrome, Lesch-Nyhan Syndrome, Leukodystrophy, Levine-Critchley Syndrome, Lewy Body Dementia, Lissencephaly, Locked-In Syndrome, Lou Gehrig's Disease, Lupus—Neurological Sequelae, Lyme Disease—Neurological Complications, Machado-Joseph Disease, Macrencephaly, Megalencephaly, Melkersson-Rosenthal Syndrome, Meningitis, Menkes Disease, Meralgia Paresthetica, Metachromatic Leukodystrophy, Microcephaly, Migraine, Miller Fisher Syndrome, Mini-Strokes, Mitochondrial Myopathies, Mobius Syndrome, Monomelic Amyotrophy, Motor Neuron Diseases, Moyamoya Disease, Mucolipidoses, Mucopolysacchari-

doses, Multi-Infarct Dementia, Multifocal Motor Neuropathy, Multiple Sclerosis, Multiple System Atrophy with Orthostatic Hypotension, Multiple System Atrophy, Muscular Dystrophy, Myasthenia—Congenital, Myasthenia Gravis, Myelinoclastic Diffuse Sclerosis, Myoclonic Encephalopathy of Infants, Myoclonus, Myopathy—Congenital, Myopathy—Thyrotoxic, Myopathy, Myotonia Congenita, Myotonia, Narcolepsy, Neuroacanthocytosis, Neurodegeneration with Brain Iron Accumulation, Neurofibromatosis, Neuroleptic Malignant Syndrome, Neurological Complications of AIDS, Neurological Manifestations of Pompe Disease, Neuromyelitis Optica, Neuromyotonia, Neuronal Ceroid Lipofuscinosis, Neuronal Migration Disorders, Neuropathy—Hereditary, Neurosarcoidosis, Neurotoxicity, Nevus Cavemosus, Niemann-Pick Disease, O'Sullivan-McLeod Syndrome, Occipital Neuralgia, Occult Spinal Dysraphism Sequence, Ohtahara Syndrome, Olivopontocerebellar Atrophy, Opsoclonus Myoclonus, Orthostatic Hypotension, Overuse Syndrome, Pain—Chronic, Paraneoplastic Syndromes, Paresthesia, Parkinson's Disease, Parmyotonia Congenita, Paroxysmal Choreoathetosis, Paroxysmal Hemicrania, Parry-Romberg, Pelizaeus-Merzbacher Disease, Pena Shokeir II Syndrome, Perineural Cysts, Periodic Paralyses, Peripheral Neuropathy, Periventricular Leukomalacia, Persistent Vegetative State, Pervasive Developmental Disorders, Phytanic Acid Storage Disease, Pick's Disease, Piriformis Syndrome, Pituitary Tumors, Polymyositis, Pompe Disease, Porencephaly, Post-Polio Syndrome, Postherpetic Neuralgia, Postinfectious Encephalomyelitis, Postural Hypotension, Postural Orthostatic Tachycardia Syndrome, Postural Tachycardia Syndrome, Primary Lateral Sclerosis, Prion Diseases, Progressive Hemifacial Atrophy, Progressive Locomotor Ataxia, Progressive Multifocal Leukoencephalopathy, Progressive Sclerosing Poliodystrophy, Progressive Supranuclear Palsy, Pseudotumor Cerebri, Pyridoxine Dependent and Pyridoxine Responsive Siezure Disorders, Ramsay Hunt Syndrome Type I, Ramsay Hunt Syndrome Type II, Rasmussen's Encephalitis and other autoimmune epilepsies, Reflex Sympathetic Dystrophy Syndrome, Refsum Disease—Infantile, Refsum Disease, Repetitive Motion Disorders, Repetitive Stress Injuries, Restless Legs Syndrome, Retrovirus-Associated Myelopathy, Rett Syndrome, Reye's Syndrome, Riley-Day Syndrome, SUNCT Headache, Sacral Nerve Root Cysts, Saint Vitus Dance, Salivary Gland Disease, Sandhoff Disease, Schilder's Disease, Schizencephaly, Seizure Disorders, Septo-Optic Dysplasia, Severe Myoclonic Epilepsy of Infancy (SMEI), Shaken Baby Syndrome, Shingles, Shy-Drager Syndrome, Sjogren's Syndrome, Sleep Apnea, Sleeping Sickness, Soto's Syndrome, Spasticity, Spina Bifida, Spinal Cord Infarction, Spinal Cord Injury, Spinal Cord Tumors, Spinal Muscular Atrophy, Spinocerebellar Atrophy, Steele-Richardson-Olszewski Syndrome, Stiff-Person Syndrome, Striatonigral Degeneration, Stroke, Sturge-Weber Syndrome, Subacute Sclerosing Panencephalitis, Subcortical Arteriosclerotic Encephalopathy, Swallowing Disorders, Sydenham Chorea, Syncope, Syphilitic Spinal Sclerosis, Syringohydromyelia, Syringomyelia, Systemic Lupus Erythematosus, Tabes Dorsalis, Tardive Dyskinesia, Tarlov Cysts, Tay-Sachs Disease, Temporal Arteritis, Tethered Spinal Cord Syndrome, Thomsen Disease, Thoracic Outlet Syndrome, Thyrotoxic Myopathy, Tic Douloureux, Todd's Paralysis, Tourette Syndrome, Transient Ischemic Attack, Transmissible Spongiform Encepha-

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lopathies, Transverse Myelitis, Traumatic Brain Injury, Tremor, Trigeminal Neuralgia, Tropical Spastic Paraparesis, Tuberous Sclerosis, Vascular Erectile Tumor, Vasculitis including Temporal Arteritis, Von Economo's Disease, Von Hippel-Lindau disease (VHL), Von Recklinghausen's Disease, Wallenberg's Syndrome, Werdnig-Hoffman Disease, Wernicke-Korsakoff Syndrome, West Syndrome, Whipple's Disease, Williams Syndrome, Wilson's Disease, X-Linked Spinal and Bulbar Muscular Atrophy, and Zellweger Syndrome.

[0357] By "respiratory disease" is meant, any disease or condition affecting the respiratory tract, such as asthma, chronic obstructive pulmonary disease or "COPD", allergic rhinitis, sinusitis, pulmonary vasoconstriction, inflammation, allergies, impeded respiration, respiratory distress syndrome, cystic fibrosis, pulmonary hypertension, pulmonary vasoconstriction, emphysema, and any other respiratory disease, condition, trait, genotype or phenotype that can respond to the modulation of disease related gene expression in a cell or tissue, alone or in combination with other therapies.

[0358] By "cardiovascular disease" is meant and disease or condition affecting the heart and vasculature, inlcuding but not limited to, coronary heart disease (CHD), cerebrovascular disease (CVD), aortic stenosis, peripheral vascular disease, atherosclerosis, arteriosclerosis, myocardial infarction (heart attack), cerebrovascular diseases (stroke), transient ischaemic attacks (TIA), angina (stable and unstable), atrial fibrillation, arrhythmia, vavular disease, congestive heart failure, hypercholoesterolemia, type I hyperlipoproteinemia, type II hyperlipoproteinemia, type III hyperlipoproteinemia, type IV hyperlipoproteinemia, type V hyperlipoproteinemia, secondary hypertrigliceridemia, and familial lecithin cholesterol acyltransferase deficiency.

[0359] By "ocular disease" as used herein is meant, any disease, condition, trait, genotype or phenotype of the eye and related structures as is known in the art, such as Cystoid Macular Edema, Asteroid Hyalosis, Pathological Myopia and Posterior Staphyloma, Toxocariasis (Ocular Larva Migrans), Retinal Vein Occlusion, Posterior Vitreous Detachment, Tractional Retinal Tears, Epiretinal Membrane, Diabetic Retinopathy, Lattice Degeneration, Retinal Vein Occlusion, Retinal Artery Occlusion, Macular Degeneration (e.g., age related macular degeneration such as wet AMD or dry AMD), Toxoplasmosis, Choroidal Melanoma, Acquired Retinoschisis, Hollenhorst Plaque, Idiopathic Central Serous Chorioretinopathy, Macular Hole, Presumed Ocular Histoplasmosis Syndrome, Retinal Macroaneursym, Retinitis Pigmentosa, Retinal Detachment, Hypertensive Retinopathy, Retinal Pigment Epithelium (RPE) Detachment, Papillophlebitis, Ocular Ischemic Syndrome, Coats' Disease, Leber's Miliary Aneurysm, Conjunctival Neoplasms, Allergic Conjunctivitis, Vernal Conjunctivitis, Acute Bacterial Conjunctivitis, Allergic Conjunctivitis &Vernal Keratoconjunctivitis, Viral Conjunctivitis, Bacterial Conjunctivitis, Chlamydial & Gonococcal Conjunctivitis, Conjunctival Laceration, Episcleritis, Scleritis, Pingueculitis, Pterygium, Superior Limbic Keratoconjunctivitis (SLK of Theodore), Toxic Conjunctivitis, Conjunctivitis with Pseudomembrane, Giant Papillary Conjunctivitis, Terrien's Marginal Degeneration, Acanthamoeba Keratitis, Fungal Keratitis, Filamentary Keratitis, Bacterial Keratitis, Keratitis Sicca/Dry Eye Syndrome, Bacterial Keratitis, Herpes Simplex Keratitis,

Sterile Corneal Infiltrates, Phlyctenulosis, Corneal Abrasion & Recurrent Corneal Erosion, Corneal Foreign Body, Chemical Burs, Epithelial Basement Membrane Dystrophy (EBMD), Thygeson's Superficial Punctate Keratopathy, Corneal Laceration, Salzmann's Nodular Degeneration, Fuchs' Endothelial Dystrophy, Crystalline Lens Subluxation, Ciliary-Block Glaucoma, Primary Open-Angle Glaucoma, Pigment Dispersion Syndrome and Pigmentary Glaucoma, Pseudoexfoliation Syndrom and Pseudoexfoliative Glaucoma, Anterior Uveitis, Primary Open Angle Glaucoma, Uveitic Glaucoma & Glaucomatocyclitic Crisis, Pigment Dispersion Syndrome & Pigmentary Glaucoma, Acute Angle Closure Glaucoma, Anterior Uveitis, Hyphema, Angle Recession Glaucoma, Lens Induced Glaucoma, Pseudoexfoliation Syndrome and Pseudoexfoliative Glaucoma, Axenfeld-Rieger Syndrome, Neovascular Glaucoma, Pars Planitis, Choroidal Rupture, Duane's Retraction Syndrome, Toxic/Nutritional Optic Neuropathy, Aberrant Regeneration of Cranial Nerve III, Intracranial Mass Lesions, Carotid-Cavernous Sinus Fistula, Anterior Ischemic Optic Neuropathy, Optic Disc Edema & Papilledema, Cranial Nerve III Palsy, Cranial Nerve IV Palsy, Cranial Nerve VI Palsy, Cranial Nerve VII (Facial Nerve) Palsy, Homer's Syndrome, Internuclear Ophthalmoplegia, Optic Nerve Head Hypoplasia, Optic Pit, Tonic Pupil, Optic Nerve Head Drusen, Demyelinating Optic Neuropathy (Optic Neuritis, Retrobulbar Optic Neuritis), Amaurosis Fugax and Transient Ischemic Attack, Pseudotumor Cerebri, Pituitary Adenoma, Molluscum Contagiosum, Canaliculitis, Verruca and Papilloma, Pediculosis and Pthiriasis, Blepharitis, Hordeolum, Preseptal Cellulitis, Chalazion, Basal Cell Carcinoma, Herpes Zoster Ophthalmicus, Pediculosis & Phthiriasis, Blowout Fracture, Chronic Epiphora, Dacryocystitis, Herpes Simplex Blepharitis, Orbital Cellulitis, Senile Entropion, and Squamous Cell Carcinoma.

[0360] By "metabolic disease" is meant any disease or condition affecting metabolic pathways as in known in the art. Metabolic disease can result in an abnormal metabolic process, either congenital due to inherited enzyme abnormality (inborn errors of metabolism) or acquired due to disease of an endocrine organ or failure of a metabolically important organ such as the liver. In one embodiment, metabolic disease includes obesity, insulin resistance, and diabetes (e.g., type I and/or type II diabetes).

[0361] By "dermatological disease" is meany any disease or condition of the skin, dermis, or any substructure therein such as hair, follicle, etc. Dermatological diseases, disorders, conditions, and traits can include psoriasis, ectopic dermatitis, skin cancers such as melanoma and basal cell carcinoma, hair loss, hair removal, alterations in pigmentation, and any other disease, condition, or trait associated with the skin, dermis, or structures therein.

[0362] By "auditory disease" is meany any disease or condition of the auditory system, including the ear, such as the inner ear, middle ear, outer ear, auditory nerve, and any substructures therein. Auditory diseases, disorders, conditions, and traits can include hearing loss, deafness, tinnitus, Meniere's Disease, vertigo, balance and motion disorders, and any other disease, condition, or trait associated with the ear, or structures therein.

[0363] In one embodiment of the present invention, each sequence of a siNA molecule of the invention is indepen-

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dently about 15 to about 30 nucleotides in length, in specific embodiments about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. In another embodiment, the siNA duplexes of the invention independently comprise about 15 to about 30 base pairs (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30). In another embodiment, one or more strands of the siNA molecule of the invention independently comprises about 15 to about 30 nucleotides (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30) that are complementary to a target nucleic acid molecule. In yet another embodiment, siNA molecules of the invention comprising hairpin or circular structures are about 35 to about 55 (e.g., about 35, 40, 45, 50 or 55) nucleotides in length, or about 38 to about 44 (e.g., about 38, 39, 40, 41, 42, 43, or 44) nucleotides in length and comprising about 15 to about 25 (e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25) base pairs.

[0364] As used herein "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism, e.g., specifically does not refer to a human. The cell can be present in an organism, e.g., birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic (e.g., bacterial cell) or eukaryotic (e.g., mammalian or plant cell). The cell can be of somatic or germ line origin, totipotent or pluripotent, dividing or non-dividing. The cell can also be derived from or can comprise a gamete or embryo, a stem cell, or a fully differentiated cell.

[0365] In one embodiment, a formulated molecular composition or formulated siNA composition of the invention is locally administered to relevant tissues ex vivo, or in vivo through direct injection, catheterization, or stenting (e.g., portal vein catherization/stenting).

[0366] In one embodiment, a formulated molecular composition or formulated siNA composition of the invention is systemically delivered to a subject or organism through parental administration as is known in the art, such as via intravenous, intramuscular, or subcutaneous injection.

[0367] In another aspect, the invention provides mammalian cells containing one or more formulated molecular composition or formulated siNA compositions of this invention. The one or more formulated molecular composition or formulated siNA compositions can independently be targeted to the same or different sites.

[0368] By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a β-D-ribofuranose moiety. The terms include double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of the instant invention can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

[0369] By "subject" is meant an organism, which is a donor or recipient of explanted cells or the cells themselves.

"Subject" also refers to an organism to which the nucleic acid molecules of the invention can be administered. A subject can be a mammal or mammalian cells, including a human or human cells.

[0370] The term "phosphorothioate" as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise a sulfur atom. Hence, the term phosphorothioate refers to both phosphorothioate and phosphorodithioate internucleotide linkages.

[0371] The term "phosphonoacetate" as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise an acetyl or protected acetyl group.

[0372] The term "thiophosphonoacetate" as used herein refers to an internucleotide linkage having Formula I, wherein Z comprises an acetyl or protected acetyl group and W comprises a sulfur atom or alternately W comprises an acetyl or protected acetyl group and Z comprises a sulfur atom.

[0373] The term "universal base" as used herein refers to nucleotide base analogs that form base pairs with each of the natural DNA/RNA bases with little discrimination between them. Non-limiting examples of universal bases include C-phenyl, C-naphthyl and other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see for example Loakes, 2001, *Nucleic Acids Research*, 29, 2437-2447).

[0374] The term "acyclic nucleotide" as used herein refers to any nucleotide having an acyclic ribose sugar, for example where any of the ribose carbons (C1, C2, C3, C4, or C5), are independently or in combination absent from the nucleotide.

[0375] In a further embodiment, the formulated molecular compositions and formulated siNA compositions can be used in combination with other known treatments to inhibit, reduce, or prevent diseases, traits, and conditions described herein or otherwise known in the art in a subject or organism. For example, the described molecules could be used in combination with one or more known compounds, treatments, or procedures to inhibit, reduce, or prevent diseases, traits, and conditions described herein or otherwise known in the art in a subject or organism. In a non-limiting example, formulated molecular composition and formulated siNA compositions that are used to treat HCV infection and comorbid conditions that are associated with HBV infection are used in combination with other HCV treatments, such as HCV vaccines; anti-HCV antibodies such as HepeX-C and Civacir; protease inhibitors such as VX-950; pegylated interferons such as PEG-Intron, and/or other antivirals such as Ribavirin and/or Valopicitabine.

[0376] In one embodiment, a formulated siNA composition of the invention comprises an expression vector comprising a nucleic acid sequence encoding at least one polynucleotide molecule of the invention (e.g., siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule) in a manner which allows expression of the siNA molecule. For example, the vector can contain sequence(s) encoding both strands of a siNA molecule comprising a duplex. The vector can also contain sequence(s) encoding a single nucleic acid molecule that is self-complementary and thus forms a siNA molecule.

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Non-limiting examples of such expression vectors are described in Paul et al., 2002, *Nature Biotechnology*, 19, 505; Miyagishi and Taira, 2002, *Nature Biotechnology*, 19, 497; Lee et al., 2002, *Nature Biotechnology*, 19, 500; and Novina et al., 2002, *Nature Medicine*, advance online publication doi:10.1038/nm725. In one embodiment, an expression vector of the invention comprises a nucleic acid sequence encoding two or more siNA molecules, which can be the same or different.

[0377] In another aspect of the invention, polynucleotides of the invention such as siNA molecules that interact with target RNA molecules and down-regulate gene encoding target RNA molecules (for example target RNA molecules referred to by Genbank Accession numbers herein) are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. Polynucleotide expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the polynucleotide molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of polynucleotide molecules. Such vectors can be repeatedly administered as necessary. For example, once expressed, the siNA molecules bind and down-regulate gene function or expression via RNA interference (RNAi). Delivery of formulated molecular compositions expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell.

[0378] By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

[0379] Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0380] FIG. 1 shows non-limiting examples of cationic lipid compounds of the invention.

[0381] FIG. 2 shows non-limiting examples of acetal linked cationic lipid compounds of the invention.

[0382] FIG. 3 shows non-limiting examples of succinyl/acyl linked cationic lipid compounds of the invention.

[0383] FIG. 4 shows non-limiting examples of aromatic cationic lipid compounds of the invention.

[0384] FIG. 5 shows non-limiting examples of additional cationic lipid compounds of the invention.

[0385] FIG. 6 shows a schematic of the components of a formulated molecular composition.

[0386] FIG. 7 shows a schematic diagram of the lamellar structure and inverted hexagonal structure that can be adopted by a formulated molecular composition.

[0387] FIG. 8 shows the components of L051, a serum-stable formulated molecular composition that undergoes a rapid pH-dependent phase transition.

[0388] FIG. 9 shows the components of L073, a serum-stable formulated molecular composition that undergoes a rapid pH-dependent phase transition.

[0389] FIG. 10 shows the components of L069, a serum-stable formulated molecular composition that undergoes a rapid pH-dependent phase transition.

[0390] FIG. 11 shows a graph depicting the serum stability of formulated molecular compositions L065, F2, L051, and L073 as determined by the relative turbidity of the formulated molecular compositions in 50% serum measured by absorbance at 500 nm. Formulated molecular compositions L065, L051, and L073 are stable in serum.

[0391] FIG. 12 shows a graph depicting the pH-dependent phase transition of formulated molecular compositions L065, F2, L051, and L073 as determined by the relative turbidity of the formulated molecular compositions in buffer solutions ranging from pH 3.5 to pH 9.0 measured by absorbance at 350 nm. Formulated molecular compositions L051 and L073 each undergo a rapid pH-dependent phase transition at pH 5.5-pH 6.5.

[0392] FIG. 13 shows a graph depicting the pH-dependent phase transition of formulated molecular composition L069 as determined by the relative turbidity of the formulated molecular composition in buffer solutions ranging from pH 3.5 to pH 9.0 measured by absorbance at 350 nm. Formulated molecular composition L069 undergoes a rapid pH-dependent phase transition at pH 5.5-pH 6.5.

[0393] FIG. 14 shows a non-limiting example of chemical modifications of siNA molecules of the invention.

[0394] FIG. 15 shows a non-limiting example of in vitro efficacy of siNA nanoparticles in reducing HBsAg levels in HepG2 cells. Active chemically modified siNA molecules were designed to target HBV site 263 RNA (siNA sequences are shown in FIG. 14). The figure shows the level of HBsAg in cells treated with formulated active siNA L051 nanoparticles (see Table IV) compared to untreated or negative control treated cells. A dose dependent reduction in HBsAg levels was observed in the active siNA treated cells, while no reduction is observed in the negative control treated cells.

[0395] FIG. 16 shows a non-limiting example of in vitro efficacy of siNA nanoparticles in reducing HBsAg levels in HepG2 cells. Active chemically modified siNA molecules were designed to target HBV site 263 RNA (siNA sequences are shown in FIG. 14). The figure shows the level of HBsAg in cells treated with formulated active siNA L053 and L054 nanoparticles (see Table IV) compared to untreated or negative control treated cells. A dose dependent reduction in HBsAg levels was observed in the active siNA treated cells, while no reduction is observed in the negative control treated cells.

[0396] FIG. 17 shows a non-limiting example of in vitro efficacy of siNA nanoparticles in reducing HBsAg levels in HepG2 cells. Active chemically modified siNA molecules were designed to target HBV site 263 RNA (siNA sequences are shown in FIG. 14). The figure shows the level of HBsAg in cells treated with formulated molecular composition L069 comprising active siNA (see Table IV) compared to untreated or negative control treated cells. A dose dependent reduction in HBsAg levels was observed in the active siNA treated cells, while no reduction is observed in the negative control treated cells.

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[0397]   **FIG. 18** shows a non-limiting example of the activity of systemically administered siNA L051 (Table IV) nanoparticles in an HBV mouse model. A hydrodynamic tail vein injection was done containing 0.3 µg of the pWTD HBV vector. The nanoparticle encapsulated active siNA molecules were administered at 3 mg/kg/day for three days via standard IV injection beginning 6 days post-HDI. Groups (N=5) of animals were sacrificed at 3 and 7 days following the last dose, and the levels of serum HBV DNA was measured. HBV DNA titers were determined by quantitative real-time PCR and expressed as mean log10 copies/ml (±SEM).

[0398]   **FIG. 19** shows a non-limiting example of the activity of systemically administered siNA L051 (Table IV) nanoparticles in an HBV mouse model. A hydrodynamic tail vein injection was done containing 0.3 µg of the pWTD HBV vector. The nanoparticle encapsulated active siNA molecules were administered at 3 mg/kg/day for three days via standard IV injection beginning 6 days post-HDI. Groups (N=5) of animals were sacrificed at 3 and 7 days following the last dose, and the levels of serum HBsAg was measured. The serum HBsAg levels were assayed by ELISA and expressed as mean log10 pg/ml (±SEM).

[0399]   **FIG. 20** shows a non-limiting example of formulated siNA L051 (Table IV) nanoparticle constructs targeting viral replication in a Huh7 HCV replicon system in a dose dependent manner. Active siNA formulatations were evaluated at 1, 5, 10, and 25 nM in comparison to untreated cells ("untreated"), and formulated inactive siNA scrambled control constructs at the same concentration.

[0400]   **FIG. 21** shows a non-limiting example of formulated siNA L053 and L054 (Table IV) nanoparticle constructs targeting viral replication in a Huh7 HCV replicon system in a dose dependent manner. Active siNA formulatations were evaluated at 1, 5, 10, and 25 nM in comparison to untreated cells ("untreated"), and formulated inactive siNA scrambled control constructs at the same concentration.

[0401]   **FIG. 22** shows the distribution of siNA in lung tissue of mice following intratracheal dosing of unformulated siNA, cholesterol-conjugated siNA, and formulated siNA (formulated molecular compositions 18.1 and 19.1). As shown, the longest half lives of exposure in lung tissue were observed with the siNA formulated in molecular compositions T018.1 or T019.1.

[0402]   **FIG. 23** shows a non-limiting example of a synthetic scheme used for the synthesis of 3-Dimethylamino-2-(Cholest-5-en-3β-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA).

[0403]   **FIG. 24** shows a non-limiting example of a synthetic scheme used for the synthesis of 1-[8'-(Cholest-5-en-3β-oxy)carboxamido-3',6'-dioxaoctanyl]carbamoyl-ω-methyl-poly(ethylene glycol) (PEG-cholesterol) and 3,4-Ditetradecoxyylbenzyl-ω-methyl-poly(ethylene glycol)ether (PEG-DMB).

[0404]   **FIG. 25** shows the components of L083, a serum-stable formulated molecular composition that undergoes a rapid pH-dependent phase transition.

[0405]   **FIG. 26** shows the components of L077, a serum-stable formulated molecular composition that undergoes a rapid pH-dependent phase transition.

[0406]   **FIG. 27** shows the components of L080, a serum-stable formulated molecular composition that undergoes a rapid pH-dependent phase transition.

[0407]   **FIG. 28** shows the components of L082, a serum-stable formulated molecular composition that undergoes a rapid pH-dependent phase transition.

[0408]   **FIG. 29** shows a non-limiting example of the activity of systemically administered siNA L077, L069, L080, L082, L083, L060, L061, and L051 (Table IV) nanoparticles in an HBV mouse model. A hydrodynamic tail vein injection was done containing 0.3 µg of the pWTD HBV vector. The nanoparticle encapsulated active siNA molecules were administered at 3 mg/kg/day for three days via standard IV injection beginning 6 days post-HDI. Groups (N=5) of animals were sacrificed at 3 and 7 days following the last dose, and the levels of serum HBV DNA was measured. HBV DNA titers were determined by quantitative real-time PCR and expressed as mean log10 copies/ml (±SEM).

[0409]   **FIG. 30** shows a non-limiting example of the dose response activity of systemically administered siNA L083 and L084 (Table IV) nanoparticles in an HBV mouse model. A hydrodynamic tail vein injection was done containing 0.3 µg of the pWTD HBV vector. The nanoparticle encapsulated active siNA molecules were administered at 3 mg/kg/day for three days via standard IV injection beginning 6 days post-HDI. Groups (N=5) of animals were sacrificed at 3 and 7 days following the last dose, and the levels of serum HBsAg was measured. The serum HBsAg levels were assayed by ELISA and expressed as mean log10 pg/ml (±SEM).

[0410]   **FIG. 31** shows a non-limiting example of the dose response activity of systemically administered siNA L077 (Table IV) nanoparticles in an HBV mouse model. A hydrodynamic tail vein injection was done containing 0.3 µg of the pWTD HBV vector. The nanoparticle encapsulated active siNA molecules were administered at 3 mg/kg/day for three days via standard IV injection beginning 6 days post-HDI. Groups (N=5) of animals were sacrificed at 3 and 7 days following the last dose, and the levels of serum HBsAg was measured. The serum HBsAg levels were assayed by ELISA and expressed as mean log10 pg/ml (±SEM).

[0411]   **FIG. 32** shows a non-limiting example of the dose response activity of systemically administered siNA L080 (Table IV) nanoparticles in an HBV mouse model. A hydrodynamic tail vein injection was done containing 0.3 µg of the pWTD HBV vector. The nanoparticle encapsulated active siNA molecules were administered at 3 mg/kg/day for three days via standard IV injection beginning 6 days post-HDI. Groups (N=5) of animals were sacrificed at 3 and 7 days following the last dose, and the levels of serum HBsAg was measured. The serum HBsAg levels were assayed by ELISA and expressed as mean log10 pg/ml (±SEM).

[0412]   **FIG. 33** shows a non-limiting example of the serum stability of siNA L077, L080, L082, and L083 (Table IV) nanoparticle formulations.

[0413]   **FIG. 34** shows a graph depicting the pH-dependent phase transition of siNA L077, L080, L082, and L083 (Table IV) nanoparticle formulations as determined by the relative turbidity of the formulated molecular composition in buffer solutions ranging from pH 3.5 to pH 9.0 measured by

absorbance at 350 nm. Formulated molecular composition L069 undergoes a rapid pH-dependent phase transition at pH 5.5-pH 6.5.

[0414] FIG. 35 shows efficacy data for LNP 58 and LNP 98 formulations targeting MapK14 site 1033 in RAW 264.7 mouse macrophage cells compared to LFK2000 and a formulated irrelevant siNA control.

[0415] FIG. 36 shows efficacy data for LNP 98 formulations targeting MapK14 site 1033 in MM14.Lu normal mouse lung cells compared to LFK2000 and a formulated irrelevant siNA control.

[0416] FIG. 37 shows efficacy data for LNP 54, LNP 97, and LNP 98 formulations targeting MapK14 site 1033 in 6.12 B lymphocyte cells compared to LFK2000 and a formulated irrelevant siNA control.

[0417] FIG. 38 shows efficacy data for LNP 98 formulations targeting MapK14 site 1033 in NIH 3T3 cells compared to LFK2000 and a formulated irrelevant siNA control.

[0418] FIG. 39 shows the dose-dependent reduction of MapK14 RNA via MapK14 LNP 54 and LNP 98 formulated siNAs in RAW 264.7 cells.

[0419] FIG. 40 shows the dose-dependent reduction of MapK14 RNA via MapK14 LNP 98 formulated siNAs in MM14.Lu cells.

[0420] FIG. 41 shows the dose-dependent reduction of MapK14 RNA via MapK14 LNP 97 and LNP 98 formulated siNAs in 6.12 B cells.

[0421] FIG. 42 shows the dose-dependent reduction of MapK14 RNA via MapK14 LNP 98 formulated siNAs in NIH 3T3 cells.

[0422] FIG. 43 shows a non-limiting example of reduced airway hyper-responsiveness from treatment with LNP-51 formulated siNAs targeting IL-4R in a mouse model of OVA challenge mediated airway hyper-responsiveness. Active formulated siNAs were tested at 0.01, 0.1, and 1.0 mg/kg and were compared to LNP vehicle along and untreated (naïve) animals.

[0423] FIG. 44 shows a non-limiting example of LNP formulated siNA mediated inhibition of huntingtin (htt) gene expression in vivo. Using Alzet osmotic pumps, siNAs encapsulated in LNPs were infused into mouse lateral ventrical or striatum for 7 or 14 days, respectively, at concentrations ranging from 0.1 to 1 mg/ml (total dose ranging from 8.4 to 84 μg). Animals treated with active siNA formulated with LNP-098 or LNP-061 were compared to mismatch control siNA formulated with LNP-061 and untreated animal controls. Huntingtin (htt) gene expression levels were determined by QPCR.

DETAILED DESCRIPTION OF THE INVENTION

Mechanism of Action of Nucleic Acid Molecules of the Invention

[0424] Aptamer: Nucleic acid aptamers can be selected to specifically bind to a particular ligand of interest (see for example Gold et al., U.S. Pat. No. 5,567,588 and U.S. Pat. No. 5,475,096, Gold et al., 1995, Annu. Rev. Biochem., 64, 763; Brody and Gold, 2000, J. Biotechnol., 74, 5; Sun, 2000,

Curr. Opin. Mol. Ther., 2, 100; Kusser, 2000, J. Biotechnol., 74, 27; Hermann and Patel, 2000, Science, 287, 820; and Jayasena, 1999, Clinical Chemistry, 45, 1628). For example, the use of in vitro selection can be applied to evolve nucleic acid aptamers with binding specificity for CylA. Nucleic acid aptamers can include chemical modifications and linkers as described herein. Nucleic apatmers of the invention can be double stranded or single stranded and can comprise one distinct nucleic acid sequence or more than one nucleic acid sequences complexed with one another. Aptamer molecules of the invention that bind to CylA, can modulate the protease activity of CylA and subsequent activation of cytolysin, and therefore modulate the acute toxicity accociated with enterococcal infection.

[0425] Antisense: Antisense molecules can be modified or unmodified RNA, DNA, or mixed polymer oligonucleotides and primarily function by specifically binding to matching sequences resulting in modulation of peptide synthesis (Wu-Pong, November 1994, BioPharm, 20-33). The antisense oligonucleotide binds to target RNA by Watson Crick base-pairing and blocks gene expression by preventing ribosomal translation of the bound sequences either by steric blocking or by activating RNase H enzyme. Antisense molecules may also alter protein synthesis by interfering with RNA processing or transport from the nucleus into the cytoplasm (Mukhopadhyay & Roth, 1996, Crit. Rev. in Oncogenesis 7, 151-190).

[0426] In addition, binding of single stranded DNA to RNA may result in nuclease degradation of the heteroduplex (Wu-Pong, supra; Crooke, supra). To date, the only backbone modified DNA chemistry which will act as substrates for RNase H are phosphorothioates, phosphorodithioates, and borontrifluoridates. Recently, it has been reported that 2'-arabino and 2'-fluoro arabino-containing oligos can also activate RNase H activity.

[0427] A number of antisense molecules have been described that utilize novel configurations of chemically modified nucleotides, secondary structure, and/or RNase H substrate domains (Woolf et al., U.S. Pat. No. 5,989,912; Thompson et al., U.S. Ser. No. 60/082,404 which was filed on Apr. 20, 1998; Hartmann et al., U.S. Ser. No. 60/101,174 which was filed on Sep. 21, 1998) all of these are incorporated by reference herein in their entirety.

[0428] Antisense DNA can be used to target RNA by means of DNA-RNA interactions, thereby activating RNase H, which digests the target RNA in the duplex. Antisense DNA can be chemically synthesized or can be expressed via the use of a single stranded DNA intracellular expression vector or the equivalent thereof.

[0429] Triplex Forming Oligonucleotides (TFO): Single stranded oligonucleotide can be designed to bind to genomic DNA in a sequence specific manner. TFOs can be comprised of pyrimidine-rich oligonucleotides which bind DNA helices through Hoogsteen Base-pairing (Wu-Pong, supra). In addition, TFOs can be chemically modified to increase binding affinity to target DNA sequences. The resulting triple helix composed of the DNA sense, DNA antisense, and TFO disrupts RNA synthesis by RNA polymerase. The TFO mechanism can result in gene expression or cell death since binding may be irreversible (Mukhopadhyay & Roth, supra)

[0430] 2'-5' Oligoadenylates: The 2-5A system is an interferon-mediated mechanism for RNA degradation found in

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higher vertebrates (Mitra et al., 1996, *Proc Nat Acad Sci USA* 93, 6780-6785). Two types of enzymes, 2-5A synthetase and RNase L, are required for RNA cleavage. The 2-5A synthetases require double stranded RNA to form 2'-5' oligoadenylates (2-5A). 2-5A then acts as an allosteric effector for utilizing RNase L, which has the ability to cleave single stranded RNA. The ability to form 2-5A structures with double stranded RNA makes this system particularly useful for modulation of viral replication.

[0431] (2'-5') oligoadenylate structures can be covalently linked to antisense molecules to form chimeric oligonucleotides capable of RNA cleavage (Torrence, supra). These molecules putatively bind and activate a 2-5A-dependent RNase, the oligonucleotide/enzyme complex then binds to a target RNA molecule which can then be cleaved by the RNase enzyme. The covalent attachment of 2'-5' oligoadenylate structures is not limited to antisense applications, and can be further elaborated to include attachment to nucleic acid molecules of the instant invention.

[0432] Enzymatic Nucleic Acid: Several varieties of naturally occurring enzymatic RNAs are presently known (Doherty and Doudna, 2001, *Annu. Rev. Biophys. Biomol. Struct.*, 30, 457-475; Symons, 1994, *Curr. Opin. Struct. Biol.*, 4, 322-30). In addition, several in vitro selection (evolution) strategies (Orgel, 1979, *Proc. R. Soc. London, B* 205, 435) have been used to evolve new nucleic acid catalysts capable of catalyzing cleavage and ligation of phosphodiester linkages (Joyce, 1989, *Gene*, 82, 83-87; Beaudry et al., 1992, *Science* 257, 635-641; Joyce, 1992, *Scientific American* 267, 90-97; Breaker et al., 1994, *TIBTECH* 12, 268; Bartel et al., 1993, *Science* 261:1411-1418; Szostak, 1993, *TIBS* 17, 89-93; Kumar et al., 1995, *FASEB J*, 9, 1183; Breaker, 1996, *Curr. Op. Biotech.*, 7, 442; Santoro et al., 1997, *Proc. Natl. Acad. Sci.*, 94, 4262; Tang et al., 1997, *RNA* 3, 914; Nakamaye & Eckstein, 1994, supra; Long & Uhlenbeck, 1994, supra; Ishizaka et al., 1995, supra; Vaish et al., 1997. *Biochemistry* 36, 6495). Each can catalyze a series of reactions including the hydrolysis of phosphodiester bonds in trans (and thus can cleave other RNA molecules) under physiological conditions.

[0433] The enzymatic nature of an enzymatic nucleic acid has significant advantages, such as the concentration of nucleic acid necessary to affect a therapeutic treatment is low. This advantage reflects the ability of the enzymatic nucleic acid molecule to act enzymatically. Thus, a single enzymatic nucleic acid molecule is able to cleave many molecules of target RNA. In addition, the enzymatic nucleic acid molecule is a highly specific modulator, with the specificity of modulation depending not only on the base-pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can be chosen to completely eliminate catalytic activity of an enzymatic nucleic acid molecule.

[0434] Nucleic acid molecules having an endonuclease enzymatic activity are able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence-specific manner. With proper design and construction, such enzymatic nucleic acid molecules can be targeted to any RNA transcript, and efficient cleavage achieved in vitro (Zaug et al., 324. *Nature* 429 1986; Uhlenbeck, 1987 *Nature* 328, 596; Kim et al., 84 *Proc. Natl. Acad. Sci. USA* 8788, 1987;

Dreyfus, 1988, *Einstein Quart. J. Bio. Med.*, 6, 92; Haseloff and Gerlach, 334 *Nature* 585, 1988; Cech, 260 *JAMA* 3030, 1988; and Jefferies et al., 17 *Nucleic Acids Research* 1371, 1989; Chartrand et al., 1995, *Nucleic Acids Research* 23, 4092; Santoro et al., 1997, *PNAS* 94, 4262).

[0435] Because of their sequence specificity, trans-cleaving enzymatic nucleic acid molecules show promise as therapeutic agents for human disease (Usman & McSwiggen, 1995 *Ann. Rep. Med. Chem.* 30, 285-294; Christoffersen and Marr, 1995 *J. Med. Chem.* 38, 2023-2037). Enzymatic nucleic acid molecule can be designed to cleave specific RNA targets within the background of cellular RNA. Such a cleavage event renders the RNA non-functional and abrogates protein expression from that RNA. In this manner, synthesis of a protein associated with a disease state can be selectively modulated (Warashina et al., 1999, *Chemistry and Biology*, 6, 237-250).

[0436] The present invention also features nucleic acid sensor molecules or allozymes having sensor domains comprising nucleic acid decoys and/or aptamers of the invention. Interaction of the nucleic acid sensor molecule's sensor domain with a molecular target can activate or inactivate the enzymatic nucleic acid domain of the nucleic acid sensor molecule, such that the activity of the nucleic acid sensor molecule is modulated in the presence of the target-signaling molecule. The nucleic acid sensor molecule can be designed to be active in the presence of the target molecule or alternately, can be designed to be inactive in the presence of the molecular target. For example, a nucleic acid sensor molecule is designed with a sensor domain comprising an aptamer with binding specificity for a ligand. In a non-limiting example, interaction of the ligand with the sensor domain of the nucleic acid sensor molecule can activate the enzymatic nucleic acid domain of the nucleic acid sensor molecule such that the sensor molecule catalyzes a reaction, for example cleavage of RNA that encodes the ligand. In this example, the nucleic acid sensor molecule is activated in the presence of ligand, and can be used as a therapeutic to treat a disease or codition associated with the ligand. Alternately, the reaction can comprise cleavage or ligation of a labeled nucleic acid reporter molecule, providing a useful diagnostic reagent to detect the presence of ligand in a system.

[0437] RNA interference: The discussion that follows discusses the proposed mechanism of RNA interference mediated by short interfering RNA as is presently known, and is not meant to be limiting and is not an admission of prior art. Applicant demonstrates herein that chemically-modified short interfering nucleic acids possess similar or improved capacity to mediate RNAi as do siRNA molecules and are expected to possess improved stability and activity in vivo; therefore, this discussion is not meant to be limiting only to siRNA and can be applied to siNA as a whole. By "improved capacity to mediate RNAi" or "improved RNAi activity" is meant to include RNAi activity measured in vitro and/or in vivo where the RNAi activity is a reflection of both the ability of the siNA to mediate RNAi and the stability of the siNAs of the invention. In this invention, the product of these activities can be increased in vitro and/or in vivo compared to an all RNA siRNA or a siNA containing a plurality of ribonucleotides. In some cases, the activity or stability of the siNA molecule can be decreased (i.e., less than ten-fold), but the overall activity of the siNA molecule is enhanced in vitro and/or in vivo.

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[0438]   RNA interference refers to the process of sequence specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire et al., 1998, *Nature*, 391, 806). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes which is commonly shared by diverse flora and phyla (Fire et al., 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response though a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2',5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

[0439]   The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as Dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Berstein et al., 2001, *Nature*, 409, 363). Short interfering RNAs derived from Dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner et al., 2001, *Science*, 293, 834). The RNAi response also features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence homologous to the siRNA. Cleavage of the target RNA takes place in the middle of the region complementary to the guide sequence of the siRNA duplex (Elbashir et al., 2001, *Genes Dev.*, 15, 188). In addition, RNA interference can also involve small RNA (e.g., micro-RNA or miRNA) mediated gene silencing, presumably though cellular mechanisms that regulate chromatin structure and thereby prevent transcription of target gene sequences (see for example Allshire, 2002, *Science*, 297, 1818-1819; Volpe et al., 2002, *Science*, 297, 1833-1837; Jenuwein, 2002, *Science*, 297, 2215-2218; and Hall et al., 2002, *Science*, 297, 2232-2237). As such, siNA molecules of the invention can be used to mediate gene silencing via interaction with RNA transcripts or alternately by interaction with particular gene sequences, wherein such interaction results in gene silencing either at the transcriptional level or post-transcriptional level.

[0440]   RNAi has been studied in a variety of systems. Fire et al., 1998, *Nature*, 391, 806, were the first to observe RNAi in *C. elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describe RNAi mediated by dsRNA in mouse embryos. Hammond et al., 2000, *Nature*, 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir et al., 2001, *Nature*, 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in *Drosophila* embryonic

lysates has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21 nucleotide siRNA duplexes are most active when containing two 2-nucleotide 3'-terminal nucleotide overhangs. Furthermore, substitution of one or both siRNA strands with 2'-deoxy or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of 3'-terminal siRNA nucleotides with deoxy nucleotides was shown to be tolerated. Mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end (Elbashir et al., 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen et al., 2001, *Cell*, 107, 309); however, siRNA molecules lacking a 5'-phosphate are active when introduced exogenously, suggesting that 5'-phosphorylation of siRNA constructs may occur in vivo.

Synthesis of Nucleic Acid Molecules

[0441]   Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs ("small" refers to nucleic acid motifs no more than 100 nucleotides in length, preferably no more than 80 nucleotides in length, and most preferably no more than 50 nucleotides in length; e.g., individual siNA oligonucleotide sequences or siNA sequences synthesized in tandem) are preferably used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of protein and/or RNA structure. Exemplary molecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

[0442]   Oligonucleotides (e.g., certain modified oligonucleotides or portions of oligonucleotides lacking ribonucleotides) are synthesized using protocols known in the art, for example as described in Caruthers et al., 1992, *Methods in Enzymology* 211, 3-19, Thompson et al., International PCT Publication No. WO 99/54459, Wincott et al., 1995, *Nucleic Acids Res.* 23, 2677-2684, Wincott et al., 1997, *Methods Mol. Bio.*, 74, 59, Brennan et al., 1998, *Biotechnol Bioeng.*, 61, 33-45, and Brennan, U.S. Pat. No. 6,001,311. All of these references are incorporated herein by reference. The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 2.5 min coupling step for 2'-O-methylated nucleotides and a 45 second coupling step for 2'-deoxy nucleotides or 2'-deoxy-2'-fluoro nucleotides. Table II outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μmol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, Calif.) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M=6.6 μmol) of 2'-O-methyl phosphoramidite and a 105-fold excess of S-ethyl tetrazole (60 μL of 0.25 M=15 μmol) can be used in

each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 22-fold excess (40 µL of 0.11 M=4.4 µmol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40 µL of 0.25 M=10 µmol) can be used in each coupling cycle of deoxy residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% N-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); and oxidation solution is 16.9 mM I$_2$, 49 mM pyridine, 9% water in THF (PerSeptive Biosystems, Inc.). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide, 0.05 M in acetonitrile) is used.

[0443] Deprotection of the DNA-based oligonucleotides is performed as follows: the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aqueous methylamine (1 mL) at 65° C. for 10 minutes. After cooling to –20° C., the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder.

[0444] The method of synthesis used for RNA including certain siNA molecules of the invention follows the procedure as described in Usman et al., 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe et al., 1990, *Nucleic Acids Res.*, 18, 5433; and Wincott et al., 1995, *Nucleic Acids Res.* 23, 2677-2684 Wincott et al., 1997, *Methods Mol. Bio.*, 74, 59, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 µmol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated nucleotides. Table II outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 µmol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, Calif.) with minimal modification to the cycle. A 33-fold excess (60 µL of 0.11 M=6.6 µmol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60 µL of 0.25 M=15 µmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 66-fold excess (120 µL of 0.11 M=13.2 µmol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120 µL of 0.25 M=30 µmol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc.

synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% N-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I$_2$, 49 mM pyridine, 9% water in THF (PerSeptive Biosystems, Inc.). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide0.05 M in acetonitrile) is used.

[0445] Deprotection of the RNA is performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65° C. for 10 min. After cooling to –20° C., the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous TEA/HF/NMP solution (300 µL of a solution of 1.5 mL N-methylpyrrolidinone, 750 µL TEA and 1 mL TEA.3HF to provide a 1.4 M HF concentration) and heated to 65° C. After 1.5 h, the oligomer is quenched with 1.5 M NH$_4$HCO$_3$.

[0446] Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65° C. for 15 minutes. The vial is brought to room temperature TEA.3HF (0.1 mL) is added and the vial is heated at 65° C. for 15 minutes. The sample is cooled at –20° C. and then quenched with 1.5 M NH$_4$HCO$_3$.

[0447] For purification of the trityl-on oligomers, the quenched NH$_4$HCO$_3$ solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is detritylated with 0.5% TFA for 13 minutes. The cartridge is then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile.

[0448] The average stepwise coupling yields are typically >98% (Wincott et al., 1995 *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted to be larger or smaller than the example described above including but not limited to 96-well format.

[0449] Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example, by ligation (Moore et al., 1992, *Science* 256, 9923; Draper et al., International PCT publication No. WO 93/23569; Shabarova et al., 1991, *Nucleic Acids Research* 19, 4247; Bellon et al., 1997, *Nucleosides & Nucleotides*, 16, 951; Bellon et al., 1997, *Bioconjugate Chem.* 8, 204), or by hybridization following synthesis and/or deprotection.

[0450] The siNA molecules of the invention can also be synthesized via a tandem synthesis methodology as

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described in Example 1 herein, wherein both siNA strands are synthesized as a single contiguous oligonucleotide fragment or strand separated by a cleavable linker which is subsequently cleaved to provide separate siNA fragments or strands that hybridize and permit purification of the siNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siNA as described herein can be readily adapted to both multiwell/multiplate synthesis platforms such as 96 well or similarly larger multi-well platforms. The tandem synthesis of siNA as described herein can also be readily adapted to large scale synthesis platforms employing batch reactors, synthesis columns and the like.

[0451]   A siNA molecule can also be assembled from two distinct nucleic acid strands or fragments wherein one fragment includes the sense region and the second fragment includes the antisense region of the RNA molecule.

[0452]   The nucleic acid molecules of the present invention can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-H (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman et al., 1994, *Nucleic Acids Symp. Ser.* 31, 163). siNA constructs can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott et al., supra, the totality of which is hereby incorporated herein by reference) and re-suspended in water.

[0453]   In another aspect of the invention, siNA molecules of the invention are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules.

Preparation of Formulated Molecular Compositions

[0454]   The method of preparation of nucleic acid formulations are disclosed in U.S. Pat. No. 5,976,567, U.S. Pat. No. 5,981,501 and PCT Patent Publication No. WO 96/40964, the teachings of all of which are incorporated in their entireties herein by reference. Cationic lipids that are useful in the present invention can be any of a number of lipid species which carry a net positive charge at a selected pH, such as physiological pH. Suitable cationic lipids include, but are not limited to, a compound having any of Formulae CLI-CLXXIX, DODAC, DOTMA, DDAB, DOTAP, DODAP, DOCDAP, DLINDAP, DOSPA, DOGS, DC-Chol and DMRIE, as well as other cationic lipids described herein, or combinations thereof. A number of these cationic lipids and related analogs, which are also useful in the present invention, have been described in U.S. Ser. No. 08/316,399; U.S. Pat. Nos. 5,208,036, 5,264,618, 5,279,833 and 5,283,185, the disclosures of which are incorporated herein by reference. Additionally, a number of commercial preparations of cationic lipids are available and can be used in the present invention. These include, for example, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, N.Y., USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANS-

FECTAM® (commercially available cationic liposomes comprising DOGS from Promega Corp., Madison, Wis., USA).

[0455]   The noncationic lipids used in the present invention can be any of a variety of neutral uncharged, zwitterionic or anionic lipids capable of producing a stable complex. They are preferably neutral, although they can alternatively be positively or negatively charged. Examples of noncationic lipids useful in the present invention include phospholipid-related materials, such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylet-hanolamine (POPE) and dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal). Noncationic lipids or sterols such as cholesterol may be present. Additional nonphosphorous containing lipids are, e.g., stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerolricinoleate, hexadecyl stereate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide and the like, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, and cerebrosides. Other lipids such as lysophosphatidylcholine and lysophosphatidylethanolamine may be present. Noncationic lipids also include polyethylene glycol-based polymers such as PEG 2000, PEG 5000 and polyethylene glycol conjugated to phospholipids or to ceramides (referred to as PEG-Cer), as described in co-pending U.S. Ser. No. 08/316, 429, incorporated herein by reference.

[0456]   In one embodiment, the noncationic lipids are diacylphosphatidylcholine (e.g., distearoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine or dilinoleoylphosphatidylcholine), diacylphosphatidylethanolamine (e.g., dioleoylphosphatidylethanolamine and palmitoyloleoylphosphatidylethanolamine), ceramide or sphingomyelin. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having about C10 to about C24 carbon chains. In one embodiment, the acyl groups are lauroyl, myristoyl, palmitoyl, stearoyl or oleoyl. In additional embodiments, the noncationic lipid comprises cholesterol, 1,2-sn-dioleoylphosphatidylethanol-amine, or egg sphingomyelin (ESM).

[0457]   In addition to cationic and neutral lipids, the formulated molecular compositions of the present invention comprise a polyethyleneglycol (PEG) conjugate. The PEG conjugate can comprise a diacylglycerol-polyethyleneglycol conjugate, i.e., a DAG-PEG conjugate. The term "diacylglycerol" refers to a compound having 2-fatty acyl chains, R1 and R2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Diacylglycerols have the following general formula VIII:

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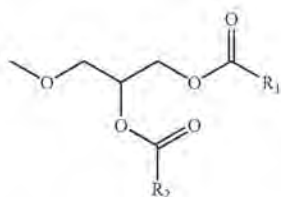
wherein R1 and R2 are each an alkyl, substituted alkyl, aryl, substituted aryl, lipid, or a ligand. In one embodiment, R1 and R2 are each independently a C2 to C30 alkyl group.

[0458] In one embodiment, the DAG-PEG conjugate is a dilaurylglycerol (C 12)-PEG conjugate, a dimyristylglycerol (C14)-PEG conjugate, a dipalmitoylglycerol (C16)-PEG conjugate, a disterylglycerol (C18)-PEG conjugate, a PEG-dilaurylglycamide conjugate (C12), a PEG-dimyristylglyca-mide conjugate (C14), a PEG-dipalmitoylglycamide conju-gate (C16), or a PEG-disterylglycamide (C18). Those of skill in the art will readily appreciate that other diacylglyc-erols can be used in the DAG-PEG conjugates of the present invention.

[0459] The PEG conjugate can alternatively comprise a conjugate other than a DAG-PEG conjugate, such as a PEG-cholesterol conjugate or a PEG-DMB conjugate.

[0460] In addition to the foregoing components, the for-mulated molecular compositions of the present invention can further comprise cationic poly(ethylene glycol) (PEG) lipids, or CPLs, that have been designed for insertion into lipid bilayers to impart a positive charge (see for example Chen, et al, 2000, *Bioconj. Chem.* 11, 433-437). Suitable formulations for use in the present invention, and methods of making and using such formulations, are disclosed, for example in U.S. application Ser. No. 09/553,639, which was filed Apr. 20, 2000, and PCT Patent Application No. CA 00/00451, which was filed Apr. 20, 2000 and which pub-lished as WO 00/62813 on Oct. 26, 2000, the teachings of each of which is incorporated herein in its entirety by reference.

[0461] The formulated molecular compositions of the present invention, i.e., those formulated molecular compo-sitions containing DAG-PEG conjugates, can be made using any of a number of different methods. For example, the lipid-nucleic acid particles can be produced via hydrophobic siNA-lipid intermediate complexes. The complexes are pref-erably charge-neutralized. Manipulation of these complexes in either detergent-based or organic solvent-based systems can lead to particle formation in which the nucleic acid is protected.

[0462] The present invention provides a method of pre-paring serum-stable formulated molecular compositions, including formulations that undergo pH-dependent phase transition, in which the biologically active molecule is encapsulated in a lipid bilayer and is protected from degra-dation. Additionally, the formulated particles formed in the present invention are preferably neutral or negatively-charged at physiological pH. For in vivo applications, neu-tral particles are advantageous, while for in vitro applica-tions the particles are more preferably negatively charged.

This provides the further advantage of reduced aggregation over the positively-charged liposome formulations in which a biologically active molecule can be encapsulated in cat-ionic lipids.

[0463] The formulated particles made by the methods of this invention have a size of about 50 to about 600 nm or more, with certain of the particles being about 65 to 85 nm. The particles can be formed by either a detergent dialysis method or by a modification of a reverse-phase method which utilizes organic solvents to provide a single phase during mixing of the components. Without intending to be bound by any particular mechanism of formation, a biologi-cally active molecule is contacted with a detergent solution of cationic lipids to form a coated molecular complex. These coated molecules can aggregate and precipitate. However, the presence of a detergent reduces this aggregation and allows the coated molecules to react with excess lipids (typically, noncationic lipids) to form particles in which the biologically active molecule is encapsulated in a lipid bilayer. The methods described below for the formation of formulated molecular compositions using organic solvents follow a similar scheme.

[0464] In some embodiments, the particles are formed using detergent dialysis. Thus, the present invention pro-vides a method for the preparation of serum-stable formu-lated molecular compositions (including formulations that undergo pH-dependent phase transition) comprising: (a) combining a molecule of interest with cationic lipids in a detergent solution to form a coated molecule-lipid complex; (b) contacting noncationic lipids with the coated molecule-lipid complex to form a detergent solution comprising a molecule-lipid complex and noncationic lipids; and (c) dialyzing the detergent solution of step (b) to provide a solution of serum-stable molecule-lipid particles, wherein the molecule of interest is encapsulated in a lipid bilayer and the particles have a size of from about 50 to about 600 nm. In one embodiment, the particles have a size of from about 50 to about 150 nm.

[0465] An initial solution of coated molecule-lipid com-plexes is formed, for example, by combining the molecule of interest with the cationic lipids in a detergent solution.

[0466] In these embodiments, the detergent solution is preferably an aqueous solution of a neutral detergent having a critical micelle concentration of 15-300 mM, more pref-erably 20-50 mM. Examples of suitable detergents include, for example, N,N'-((octanoylimino)-bis-(trimethylene))-bis-(D-gluconamide) (BIGCHAP); BRIJ 35; Deoxy-BIGC-HAP; dodecylpoly(ethylene glycol) ether; Tween 20; Tween 40; Tween 60; Tween 80; Tween 85; Mega 8; Mega 9; Zwittergent® 3-08; Zwittergent® 3-10; Triton X-405; hexyl-, heptyl-, octyl- and nonyl-beta-D-glucopyranoside; and heptylthioglucopyranoside. In one embodiment, the detergent is octyl β-D-glucopyranoside or Tween-20. The concentration of detergent in the detergent solution is typi-cally about 100 mM to about 2 M, preferably from about 200 mM to about 1.5 M.

[0467] The cationic lipids and molecules to be encapsu-lated will typically be combined to produce a charge ratio (+/−) of about 1:1 to about 20:1, preferably in a ratio of about 1:1 to about 12:1, and more preferably in a ratio of about 2:1 to about 6:1. Additionally, the overall concentra-

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tion of the molecules of interest in solution will typically be from about 25 μg/mL to about 1 mg/mL, preferably from about 25 μg/mL to about 500 μg/mL, and more preferably from about 100 μg/mL to about 250 μg/mL. The combination of molecules and cationic lipids in detergent solution is kept, typically at room temperature, for a period of time which is sufficient for the coated complexes to form. Alternatively, the molecules and cationic lipids can be combined in the detergent solution and warmed to temperatures of up to about 37° C. For molecules which are particularly sensitive to temperature, the coated complexes can be formed at lower temperatures, typically down to about 4° C.

[0468] In one embodiment, the molecule to lipid ratios (mass/mass ratios) in a formed formulated molecular composition will range from about 0.01 to about 0.08. The ratio of the starting materials also falls within this range because the purification step typically removes the unencapsulated molecule as well as the empty liposomes. In another embodiment, the formulated molecular composition preparation uses about 400 μg siNA per 10 mg total lipid or a molecule to lipid ratio of about 0.01 to about 0.08 and, more preferably, about 0.04, which corresponds to 1.25 mg of total lipid per 50 μg of siNA.

[0469] The detergent solution of the coated molecule-lipid complexes is then contacted with neutral lipids to provide a detergent solution of molecule-lipid complexes and neutral lipids. The neutral lipids which are useful in this step include, among others, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cardiolipin, and cerebrosides. In preferred embodiments, the neutral lipids are diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide or sphingomyelin. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C10-C24 carbon chains. More preferably the acyl groups are lauroyl, myristoyl, palmitoyl, stearoyl or oleoyl. In preferred embodiments, the neutral lipid is 1,2-sn-dioleoylphosphatidylethanolamine (DOPE), palmitoyl oleoyl phosphatidylcholine (POPC), egg phosphatidylcholine (EPC), distearoylphosphatidylcholine (DSPC), cholesterol, or a mixture thereof. In the most preferred embodiments, the siNA-lipid particles are fusogenic particles with enhanced properties in vivo and the neutral lipid is DSPC or DOPE. As explained above, the siNA-lipid particles of the present invention can further comprise PEG conjugates, such as DAG-PEG conjugates, PEG-cholesterol conjugates, and PEG-DMB conjugates. In addition, the siNA-lipid particles of the present invention can further comprise cholesterol.

[0470] The amount of neutral lipid which is used in the present methods is typically about 0.5 to about 10 mg of total lipids to 50 μg of the molecule of interest. Preferably the amount of total lipid is from about 1 to about 5 mg per 50 μg of the molecule of interest.

[0471] Following formation of the detergent solution of molecule-lipid complexes and neutral lipids, the detergent is removed, preferably by dialysis. The removal of the detergent results in the formation of a lipid-bilayer which surrounds the molecule of interest providing serum-stable molecule-lipid particles which have a size of from about 50 nm to about 150 or 50 nm to about 600 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0472] The serum-stable molecule-lipid particles can be sized by any of the methods available for sizing liposomes as are known in the art. The sizing can be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

[0473] Several techniques are available for sizing the particles to a desired size. One sizing method, used for liposomes and equally applicable to the present particles is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a particle suspension either by bath or probe sonication produces a progressive size reduction down to particles of less than about 50 nm in size. Homogenization is another method which relies on shearing energy to fragment larger particles into smaller ones. In a typical homogenization procedure, particles are recirculated through a standard emulsion homogenizer until selected particle sizes, typically between about 60 and 80 nm, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination, or QELS.

[0474] Extrusion of the particles through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing particle sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired particle size distribution is achieved. The particles can be extruded through successively smaller-pore membranes, to achieve a gradual reduction in size.

[0475] In another group of embodiments, the present invention provides a method for the preparation of a formulated molecular composition, comprising: (a) preparing a mixture comprising cationic lipids and noncationic lipids in an organic solvent; (b) contacting an aqueous solution of molecule of interest with the mixture in step (a) to provide a clear single phase; and (c) removing the organic solvent to provide a suspension of molecule-lipid particles, wherein the molecule of interest is encapsulated in a lipid bilayer, and the particles are stable in serum and have a size of from about 50 to about 150 nm or alternately 50 to about 600 nm.

[0476] The molecules of interest, cationic lipids and noncationic lipids which are useful in this group of embodiments are as described for the detergent dialysis methods above.

[0477] The selection of an organic solvent will typically involve consideration of solvent polarity and the ease with which the solvent can be removed at the later stages of particle formation. The organic solvent, which is also used as a solubilizing agent, is in an amount sufficient to provide a clear single phase mixture of biologically active molecules and lipids. Suitable solvents include, but are not limited to, chloroform, dichloromethane, diethylether, cyclohexane, cyclopentane, benzene, toluene, methanol, or other aliphatic alcohols such as propanol, isopropanol, butanol, tert-butanol, iso-butanol, pentanol and hexanol. Combinations of two or more solvents can also be used in the present invention.

[0478] Contacting the molecules of interest with the organic solution of cationic and neutral lipids is accomplished by mixing together a first solution of the molecule of interest, which is typically an aqueous solution, and a second organic solution of the lipids. One of skill in the art will

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understand that this mixing can take place by any number of methods, for example by mechanical means such as by using vortex mixers.

[0479] After the molecule of interest has been contacted with the organic solution of lipids, the organic solvent is removed, thus forming an aqueous suspension of serum-stable molecule-lipid particles. The methods used to remove the organic solvent will typically involve evaporation at reduced pressures or blowing a stream of inert gas (e.g., nitrogen or argon) across the mixture.

[0480] The formulated molecular compositions thus formed will typically be sized from about 50 nm to 150 nm or alternately from about 50 nm to 600 nm. To achieve further size reduction or homogeneity of size in the particles, sizing can be conducted as described above.

[0481] In other embodiments, the methods will further comprise adding nonlipid polycations which are useful to effect the transformation of cells using the present compositions. Examples of suitable nonlipid polycations include, but are limited to, hexadimethrine bromide (sold under the brandname POLYBRENE®, from Aldrich Chemical Co., Milwaukee, Wis., USA) or other salts of hexadimethrine. Other suitable polycations include, for example, salts of poly-L-ornithine, poly-L-arginine, poly-L-lysine, poly-D-lysine, polyallylamine and polyethyleneimine.

[0482] In certain embodiments, the formation of the formulated molecular compositions can be carried out either in a mono-phase system (e.g., a Bligh and Dyer monophase or similar mixture of aqueous and organic solvents) or in a two-phase system with suitable mixing.

[0483] When formation of the complexes is carried out in a mono-phase system, the cationic lipids and molecules of interest are each dissolved in a volume of the mono-phase mixture. Combination of the two solutions provides a single mixture in which the complexes form. Alternatively, the complexes can form in two-phase mixtures in which the cationic lipids bind to the molecule (which is present in the aqueous phase), and "pull" it into the organic phase.

[0484] In another embodiment, the present invention provides a method for the preparation of formulated molecular composition, comprising: (a) contacting molecules of interest with a solution comprising noncationic lipids and a detergent to form a molecule-lipid mixture; (b) contacting cationic lipids with the molecule-lipid mixture to neutralize a portion of the negative charge of the molecule of interest and form a charge-neutralized mixture of molecules and lipids; and (c) removing the detergent from the charge-neutralized mixture to provide the formulated molecular composition.

[0485] In one group of embodiments, the solution of neutral lipids and detergent is an aqueous solution. Contacting the molecules of interest with the solution of neutral lipids and detergent is typically accomplished by mixing together a first solution of the molecule of interst and a second solution of the lipids and detergent. One of skill in the art will understand that this mixing can take place by any number of methods, for example, by mechanical means such as by using vortex mixers. Preferably, the molecule solution is also a detergent solution. The amount of neutral lipid which is used in the present method is typically determined based on the amount of cationic lipid used, and is typically

of from about 0.2 to 5 times the amount of cationic lipid, preferably from about 0.5 to about 2 times the amount of cationic lipid used.

[0486] The molecule-lipid mixture thus formed is contacted with cationic lipids to neutralize a portion of the negative charge which is associated with the molecule of interest (or other polyanionic materials) present. The amount of cationic lipids used is typically the amount sufficient to neutralize at least 50% of the negative charge of the molecule of interest. Preferably, the negative charge will be at least 70% neutralized, more preferably at least 90% neutralized. Cationic lipids which are useful in the present invention include, for example, compounds having any of formulae CLI-CLXXIX, DODAC, DOTMA, DDAB, DOTAP, DC-Chol, DMOBA, CLinDMA, and DMRIE. These lipids and related analogs have been described in U.S. Ser. No. 08/316,399; U.S. Pat. Nos. 5,208,036, 5,264,618, 5,279,833 and 5,283,185, the disclosures of which are incorporated by reference in their entireties herein. Additionally, a number of commercial preparations of cationic lipids are available and can be used in the present invention. These include, for example, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, N.Y., USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic lipids comprising DOGS in ethanol from Promega Corp., Madison, Wis., USA).

[0487] Contacting the cationic lipids with the molecule-lipid mixture can be accomplished by any of a number of techniques, preferably by mixing together a solution of the cationic lipid and a solution containing the molecule-lipid mixture. Upon mixing the two solutions (or contacting in any other manner), a portion of the negative charge associated with the molecule of interest is neutralized.

[0488] After the cationic lipids have been contacted with the molecule-lipid mixture, the detergent (or combination of detergent and organic solvent) is removed, thus forming the formulated molecular composition. The methods used to remove the detergent typically involve dialysis. When organic solvents are present, removal is typically accomplished by evaporation at reduced pressures or by blowing a stream of inert gas (e.g., nitrogen or argon) across the mixture.

[0489] The formulated molecular composition particles thus formed is typically sized from about 50 nm to several microns. To achieve further size reduction or homogeneity of size in the particles, the formulated molecular composition particles can be sonicated, filtered or subjected to other sizing techniques which are used in liposomal formulations and are known to those of skill in the art.

[0490] In other embodiments, the methods further comprise adding nonlipid polycations which are useful to affect the lipofection of cells using the present compositions. Examples of suitable nonlipid polycations include, hexadimethrine bromide (sold under the brandname POLYBRENE®, from Aldrich Chemical Co., Milwaukee, Wis., USA) or other salts of hexadimethrine. Other suitable polycations include, for example, salts of poly-L-ornithine, poly-L-arginine, poly-L-lysine, poly-D-lysine, polyallylamine and polyethyleneimine. Addition of these salts is preferably after the particles have been formed.

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[0491] In another aspect, the present invention provides methods for the preparation of formulated siNA compositions, comprising: (a) contacting an amount of cationic lipids with siNA in a solution; the solution comprising from about 15-35% water and about 65-85% organic solvent and the amount of cationic lipids being sufficient to produce a +/–charge ratio of from about 0.85 to about 2.0, to provide a hydrophobic lipid-siNA complex; (b) contacting the hydrophobic, lipid-siNA complex in solution with neutral lipids, to provide a siNA-lipid mixture; and (c) removing the organic solvents from the lipid-siNA mixture to provide formulated siNA composition particles.

[0492] The siNA, neutral lipids, cationic lipids and organic solvents which are useful in this aspect of the invention are the same as those described for the methods above which used detergents. In one group of embodiments, the solution of step (a) is a mono-phase. In another group of embodiments, the solution of step (a) is two-phase.

[0493] In one embodiment, the cationic lipids used in a formulation of the invention are selected from a compound having Formula CLI, CLII, CLIII, CLIV, CLV, CLVI, CLVII, CLVIII, CLIX, CLX, CLXI, CLXII, CLXIII, CLXIV, CLXV, CLXVI, CLXVII, CLXVIII, CLXIX, CLXX, CLXXI, CLXXII, CLXXIII, CLXXIV, CLXXV, CLXXVI, CLXXVII, CLXXVIII, CLXXIX, and DODAC, DDAB, DOTMA, DODAP, DOCDAP, DLINDAP, DOSPA, DMRIE, DOGS, DMOBA, CLinDMA, and combinations thereof. In one embodiment, the noncationic lipids are selected from ESM, DOPE, DOPC, DSPC, polyethylene glycol-based polymers (e.g., PEG 2000, PEG 5000 or PEG-modified diacylglycerols), distearoylphosphatidylcholine (DSPC), cholesterol, and combinations thereof. In one embodiment, the organic solvents are selected from methanol, chloroform, methylene chloride, ethanol, diethyl ether and combinations thereof.

[0494] In one embodiment, the cationic lipid is a compound having Formula CLI, CLII, CLIII, CLIV, CLV, CLVI, CLVII, CLVIII, CLIX, CLX, CLXI, CLXII, CLXIII, CLXIV, CLXV, CLXVI, CLXVII, CLXVIII, CLXVII, CLXVIII, CLXIX, CLXX, CLXXI, CLXXII, CLXXIII, CLXXIV, CLXXV, CLXXVI, CLXXVII, CLXXVIII, CLXXIX or DODAC, DOTAP, DODAP, DOCDAP, DLINDAP, DDAB, DOTMA, DOSPA, DMRIE, DOGS or combinations thereof; the noncationic lipid is ESM, DOPE, DAG-PEGs, distearoylphosphatidylcholine (DSPC), cholesterol, or combinations thereof (e.g. DSPC and DAG-PEGs); and the organic solvent is methanol, chloroform, methylene chloride, ethanol, diethyl ether or combinations thereof.

[0495] As above, contacting the siNA with the cationic lipids is typically accomplished by mixing together a first solution of siNA and a second solution of the lipids, preferably by mechanical means such as by using vortex mixers. The resulting mixture contains complexes as described above. These complexes are then converted to particles by the addition of neutral lipids and the removal of the organic solvent. The addition of the neutral lipids is typically accomplished by simply adding a solution of the neutral lipids to the mixture containing the complexes. A reverse addition can also be used. Subsequent removal of organic solvents can be accomplished by methods known to those of skill in the art and also described above.

[0496] The amount of neutral lipids which is used in this aspect of the invention is typically an amount of from about 0.2 to about 15 times the amount (on a mole basis) of cationic lipids which was used to provide the charge-neutralized lipid-nucleic acid complex. Preferably, the amount is from about 0.5 to about 9 times the amount of cationic lipids used.

[0497] In yet another aspect, the present invention provides formulated siNA compositions which are prepared by the methods described above. In these embodiments, the formulated siNA compositions are either net charge neutral or carry an overall charge which provides the formulated siNA compositions with greater lipofection activity. In one embodiment, the noncationic lipid is egg sphingomyelin and the cationic lipid is DODAC. In one embodiment, the noncationic lipid is a mixture of DSPC and cholesterol, and the cationic lipid is DOTMA. In another embodiment, the noncationic lipid can further comprise cholesterol.

[0498] A variety of general methods for making formulated siNA composition-CPLs (CPL-containing formulated siNA compositions) are discussed herein. Two general techniques include "post-insertion" technique, that is, insertion of a CPL into for example, a preformed formulated siNA composition, and the "standard" technique, wherein the CPL is included in the lipid mixture during for example, the formulated siNA composition formation steps. The post-insertion technique results in formulated siNA compositions having CPLs mainly in the external face of the formulated siNA composition bilayer membrane, whereas standard techniques provide formulated siNA compositions having CPLs on both internal and external faces.

[0499] In particular, "post-insertion" involves forming formulated siNA compositions (by any method), and incubating the pre-formed formulated siNA compositions in the presence of CPL under appropriate conditions (preferably 2-3 hours at 60° C.). Between 60-80% of the CPL can be inserted into the external leaflet of the recipient vesicle, giving final concentrations up to about 5 to 10 mol % (relative to total lipid). The method is especially useful for vesicles made from phospholipids (which can contain cholesterol) and also for vesicles containing PEG-lipids (such as PEG-DAGs).

[0500] In an example of a "standard" technique, the CPL-formulated siNA compositions of the present invention can be formed by extrusion. In this embodiment, all of the lipids including the CPL, are co-dissolved in chloroform, which is then removed under nitrogen followed by high vacuum. The lipid mixture is hydrated in an appropriate buffer, and extruded through two polycarbonate filters with a pore size of 100 nm. The resulting formulated siNA compositions contain CPL on both of the internal and external faces. In yet another standard technique, the formation of CPL-formulated siNA compositions can be accomplished using a detergent dialysis or ethanol dialysis method, for example, as discussed in U.S. Pat. Nos. 5,976,567 and 5,981,501, both of which are incorporated by reference in their entireties herein.

[0501] The formulated siNA compositions of the present invention can be administered either alone or in mixture with a physiologically-acceptable carrier (such as physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice. Generally, normal saline will be employed as the pharmaceutically acceptable carrier. Other suitable carriers

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include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, etc.

[0502] The pharmaceutical carrier is generally added following formulated siNA composition formation. Thus, after the formulated siNA composition is formed, the formulated siNA composition can be diluted into pharmaceutically acceptable carriers such as normal saline.

[0503] The concentration of formulated siNA compositions in the pharmaceutical formulations can vary widely, i.e., from less than about 0.05%, usually at or at least about 2-5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. For example, the concentration can be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, formulated siNA compositions composed of irritating lipids can be diluted to low concentrations to lessen inflammation at the site of administration.

[0504] As described above, the formulated siNA compositions of the present invention comprise DAG-PEG conjugates. It is often desirable to include other components that act in a manner similar to the DAG-PEG conjugates and that serve to prevent particle aggregation and to provide a means for increasing circulation lifetime and increasing the delivery of the formulated siNA compositions to the target tissues. Such components include, but are not limited to, PEG-lipid conjugates, such as PEG-ceramides or PEG-phospholipids (such as PEG-PE), ganglioside GM1-modified lipids or ATTA-lipids to the particles. Typically, the concentration of the component in the particle will be about 1-20% and, more preferably from about 3-10%.

[0505] The pharmaceutical compositions of the present invention can be sterilized by conventional, well known sterilization techniques. Aqueous solutions can be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, and calcium chloride. Additionally, the particle suspension can include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable

[0506] In another example of their use, formulated molecular compositions can be incorporated into a broad range of topical dosage forms including, but not limited to, gels, oils, emulsions and the like. For instance, the suspension containing the formulated molecular compositions can be formulated and administered as topical creams, pastes, ointments, gels, lotions and the like.

[0507] Once formed, the formulated molecular compositions of the present invention are useful for the introduction of biologically active molecules into cells. Accordingly, the

present invention also provides methods for introducing a biologically active molecule into a cell. The methods are carried out in vitro or in vivo by first forming the formulated molecular compositions as described above and then contacting the formulated molecular compositions with the cells for a period of time sufficient for transfection to occur.

[0508] The formulated molecular compositions of the present invention can be adsorbed to almost any cell type with which they are mixed or contacted. Once adsorbed, the formulations can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the biologically acitive molecule portion of the formulation can take place via any one of these pathways. In particular, when fusion takes place, the particle membrane is integrated into the cell membrane and the contents of the particle, i.e., biologically active molecules, combine with the intracellular fluid, for example, the cytoplasm. The serum stable formulated molecular compositions that undergo pH-dependent phase transition demonstrate an increase in cell fusion at early endosomal pH (i.e., about pH 5.5-6.5), resulting in efficient delivery of the contents of the particle, i.e., biologically active molecules, to the cell.

[0509] Using the Endosomal Release Parameter (ERP) assay of the present invention, the transfection efficiency of the formulated molecular composition or other lipid-based carrier system can be optimized. More particularly, the purpose of the ERP assay is to distinguish the effect of various cationic lipids and helper lipid components of formulated molecular compositions based on their relative effect on binding/uptake or fusion with/destabilization of the endosomal membrane. This assay allows one to determine quantitatively how each component of the formulated molecular composition or other lipid-based carrier system effects transfection efficacy, thereby optimizing the formulated molecular compositions or other lipid-based carrier systems. As explained herein, the Endosomal Release Parameter or, alternatively, ERP is defined as: Reporter Gene Expression/Cell divided by formulated molecular composition Uptake/Cell.

[0510] It will be readily apparent to those of skill in the art that any reporter gene (e.g., luciferase, beta-galactosidase, green fluorescent protein, etc.) can be used in the assay. In addition, the lipid component (or, alternatively, any component of the formulated molecular composition) can be labeled with any detectable label provided the does inhibit or interfere with uptake into the cell. Using the ERP assay of the present invention, one of skill in the art can assess the impact of the various lipid components (e.g., cationic lipid, neutral lipid, PEG-lipid derivative, PEG-DAG conjugate, ATTA-lipid derivative, calcium, CPLs, cholesterol, etc.) on cell uptake and transfection efficiencies, thereby optimizing the formulated siNA composition. By comparing the ERPs for each of the various formulated molecular compositions, one can readily determine the optimized system, e.g., the formulated molecular composition that has the greatest uptake in the cell coupled with the greatest transfection efficiency.

[0511] Suitable labels for carrying out the ERP assay of the present invention include, but are not limited to, spectral labels, such as fluorescent dyes (e.g., fluorescein and derivatives, such as fluorescein isothiocyanate (FITC) and Oregon

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Green9; rhodamine and derivatives, such Texas red, tetrarhodimine isothiocynate (TRITC), etc., digoxigenin, biotin, phycoerythrin, AMCA, CyDyes, and the like; radiolabels, such as ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, etc.; enzymes, such as horse radish peroxidase, alkaline phosphatase, etc.; spectral calorimetric labels, such as colloidal gold or colored glass or plastic beads, such as polystyrene, polypropylene, latex, etc. The label can be coupled directly or indirectly to a component of the formulated molecular composition using methods well known in the art. As indicated above, a wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with the formulated siNA composition, stability requirements, and available instrumentation and disposal provisions.

[0512]   In addition, the transfection efficiency of the formulated molecular composition or other lipid-based carrier system can be determined by measuring the stability of the composition in serm and/or measuring the pH dependent phase transition of the formulated molecular composition, wherein a determination that the formulated molecular composition is stable in serum and a determination that the formulated molecular composition undergoes a phase transition at about pH 5.5-6.5 indicates that the formulated molecular composition will have increased transfection efficiency. The serum stability of the formulated molecular composition can be measured using, for example, an assay that measures the relative turbidity of the composition in serum and determining that the turbity of the composition in serum remains constant over time. The pH dependent phase transition of the formulated molecular composition can be measured using an assay that measures the relative turbidity of the composition at different pH over time and determining that the turbidity changes when the pH differs from physiologic pH.

Optimizing Activity of the Nucleic Acid Molecule of the Invention.

[0513]   Chemically synthesizing nucleic acid molecules (e.g., siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule) with modifications (base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency (see e.g., Eckstein et al., International Publication No. WO 92/07065; Perrault et al., 1990 *Nature* 344, 565; Pieken et al., 1991, *Science* 253, 314; Usman and Cedergren, 1992, *Trends in Biochem. Sci.* 17, 334; Usman et al., International Publication No. WO 93/15187; and Rossi et al., International Publication No. WO 91/03162; Sproat, U.S. Pat. No. 5,334,711; Gold et al., U.S. Pat. No. 6,300,074; and Burgin et al., supra; all of which are incorporated by reference herein). All of the above references describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein. Modifications that enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

[0514]   There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease

resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-O-allyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, *TIBS.* 17, 34; Usman et al., 1994, *Nucleic Acids Symp. Ser.* 31, 163; Burgin et al., 1996, *Biochemistry,* 35, 14090). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein et al., International Publication PCT No. WO 92/07065; Perrault et al. *Nature,* 1990, 344, 565-568; Pieken et al. *Science,* 1991, 253, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.,* 1992, 17, 334-339; Usman et al. International Publication PCT No. WO 93/15187; Sproat, U.S. Pat. No. 5,334,711 and Beigelman et al., 1995, *J. Biol. Chem.,* 270, 25702; Beigelman et al., International PCT publication No. WO 97/26270; Beigelman et al., U.S. Pat. No. 5,716,824; Usman et al., U.S. Pat. No. 5,627,053; Woolf et al., International PCT Publication No. WO 98/13526; Thompson et al., U.S. Ser. No. 60/082,404 which was filed on Apr. 20, 1998; Karpeisky et al., 1998, *Tetrahedron Lett.,* 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic Acid Sciences),* 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.,* 67, 99-134; and Burlina et al., 1997, *Bioorg. Med. Chem.,* 5, 1999-2010; all of the references are hereby incorporated in their totality by reference herein). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid molecules without modulating catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the siNA nucleic acid molecules of the instant invention so long as the ability of siNA to promote RNAi cells is not significantly inhibited.

[0515]   While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorodithioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity or decreased activity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and higher specificity of these molecules.

[0516]   Polynucleotides (e.g., siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule) having chemical modifications that maintain or enhance activity are provided. Such a nucleic acid is also generally more resistant to nucleases than an unmodified nucleic acid. Accordingly, the in vitro and/or in vivo activity should not be significantly lowered. In cases in which modulation is the goal, therapeutic nucleic acid molecules delivered exogenously should optimally be stable within cells until translation of the target RNA has been modulated long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Improvements in the chemical synthesis of RNA and DNA (Wincott et al., 1995, *Nucleic Acids Res.* 23, 2677; Caruthers et al., 1992, *Methods in Enzymology* 211, 3-19 (incorporated by reference herein)) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability, as described above.

[0517]   In one embodiment, nucleic acid molecules of the invention include one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) G-clamp nucleotides. A G-clamp nucle-

otide is a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example Lin and Matteucci, 1998, *J. Am. Chem. Soc.*, 120, 8531-8532. A single G-clamp analog substitution within an oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such nucleotides in nucleic acid molecules of the invention results in both enhanced affinity and specificity to nucleic acid targets, complementary sequences, or template strands. In another embodiment, nucleic acid molecules of the invention include one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) LNA "locked nucleic acid" nucleotides such as a 2',4'-C methylene bicyclo nucleotide (see for example Wengel et al., International PCT Publication No. WO 00/66604 and WO 99/14226).

[0518] In another embodiment, the invention features conjugates and/or complexes of siNA molecules of the invention. Such conjugates and/or complexes can be used to facilitate delivery of siNA molecules into a biological system, such as a cell. The conjugates and complexes provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid molecules of the invention. The present invention encompasses the design and synthesis of novel conjugates and complexes for the delivery of molecules, including, but not limited to, small molecules, lipids, cholesterol, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for example proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across cellular membranes. In general, the transporters described are designed to be used either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid molecules of the invention into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, U.S. Pat. No. 5,854,038). Conjugates of the molecules described herein can be attached to biologically active molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

[0519] The term "biodegradable linker" as used herein, refers to a nucleic acid or non-nucleic acid linker molecule that is designed as a biodegradable linker to connect one molecule to another molecule, for example, a biologically active molecule to a siNA molecule of the invention or the sense and antisense strands of a siNA molecule of the invention. The biodegradable linker is designed such that its stability can be modulated for a particular purpose, such as delivery to a particular tissue or cell type. The stability of a nucleic acid-based biodegradable linker molecule can be modulated by using various chemistries, for example combinations of ribonucleotides, deoxyribonucleotides, and chemically-modified nucleotides, such as 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus-based link-

age, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

[0520] The term "biodegradable" as used herein, refers to degradation in a biological system, for example, enzymatic degradation or chemical degradation.

[0521] The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phosphorus-containing group and saturated or unsaturated alkyl group, optionally substituted with OH, COOH, oxo, amine, or substituted or unsubstituted aryl groups.

[0522] Therapeutic nucleic acid molecules (e.g., siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule) delivered exogenously optimally are stable within cells until reverse transcription of the RNA has been modulated long enough to reduce the levels of the RNA transcript. The nucleic acid molecules are resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of nucleic acid molecules described in the instant invention and in the art have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

[0523] In yet another embodiment, siNA molecules having chemical modifications that maintain or enhance enzymatic activity of proteins involved in RNAi are provided. Such nucleic acids are also generally more resistant to nucleases than unmodified nucleic acids. Thus, in vitro and/or in vivo the activity should not be significantly lowered.

[0524] Use of the nucleic acid-based molecules of the invention will lead to better treatments by affording the possibility of combination therapies (e.g., multiple siNA molecules targeted to different genes; nucleic acid molecules coupled with known small molecule modulators; or intermittent treatment with combinations of molecules, including different motifs and/or other chemical or biological molecules).

[0525] In another aspect a polynucleotide molecule of the invention (e.g., siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucleotide, or other nucleic acid molecule) comprises one or more 5' and/or a 3'-cap structure, for example, on only the sense siNA strand, the antisense siNA strand, or both siNA strands.

[0526] By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Adamic et al., U.S. Pat. No. 5,998,203, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or localization within a cell. The cap may be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or may be present on both termini. In non-limiting examples, the 5'-cap includes, but is not limited to, glyceryl, inverted deoxy abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; threo-pentofuranosyl nucle-

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otide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxy-butyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety

[0527] Non-limiting examples of the 3'-cap include, but are not limited to, glyceryl, inverted deoxy abasic residue (moiety), 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; threo-pento-furanosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-di-hydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butane-diol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phospho-rodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, Tetrahedron 49, 1925; incorporated by reference herein).

[0528] By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine and therefore lacks a base at the 1'-position.

[0529] An "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, and unless expressly stated to to the contrary, the alkyl group has 1 to 12 carbons. More preferably, it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO$_2$ or N(CH$_3$)$_2$, amino, or SH. The term also includes alkenyl groups that are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably, it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group may be substituted or unsub-stituted. When substituted the substituted group(s) is pref-erably, hydroxyl, cyano, alkoxy, =O, =S, NO$_2$, halogen, N(CH$_3$)$_2$, amino, or SH. The term "alkyl" also includes alkynyl groups that have an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Prefer-ably, the alkynyl group has 1 to 12 carbons. More preferably, it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO$_2$ or N(CH$_3$)$_2$, amino or SH.

[0530] Such alkyl groups can also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. An "aryl" group refers to an aromatic group that has at least one ring having a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. The preferred substitu-ent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Hetero-cyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazi-nyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an —C(O)—NH—R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an —C(O)—OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

[0531] By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchange-ably as nucleotide analogs, modified nucleotides, non-natu-ral nucleotides, non-standard nucleotides and other; see, for example, Usman and McSwiggen, supra; Eckstein et al., International PCT Publication No. WO 92/07065; Usman et al, International PCT Publication No. WO 93/15187; Uhl-man & Peyman, supra, all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach et al., 1994, Nucleic Acids Res. 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2,4,6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g., 5-methylcy-tidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyri-midines (e.g. 6-methyluridine), propyne, and others (Burgin et al., 1996, Biochemistry, 35, 14090; Uhlman & Peyman, supra). By "modified bases" in this aspect is meant nucle-otide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents.

[0532] In one embodiment, the invention features modi-fied polynucleotide molecules (e.g., siNA, antisense, aptamer, decoy, ribozyme, 2-5A, triplex forming oligonucle-otide, or other nucleic acid molecule), with phosphate back-bone modifications comprising one or more phosphorothio-ate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate carbamate, carboxym-ethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and/or alkylsilyl, sub-stitutions. For a review of oligonucleotide backbone modi-fications, see Hunziker and Leumann, 1995, Nucleic Acid Analogues: Synthesis and Properties, in Modern Synthetic Methods, VCH, 331-417, and Mesmaeker et al., 1994, Novel

Backbone Replacements for Oligonucleotides, in *Carbohydrate Modifications in Antisense Research*, ACS, 24-39.

[0533] By "abasic" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, see for example Adamic et al., U.S. Pat. No. 5,998,203.

[0534] By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine, thymine, or uracil joined to the 1' carbon of β-D-ribo-furanose.

[0535] By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate. Non-limiting examples of modified nucleotides are shown by Formulae I-VII and/or other modifications described herein.

[0536] In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH2 or 2'-O—NH$_2$, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein et al., U.S. Pat. No. 5,672,695 and Matulic-Adamic et al., U.S. Pat. No. 6,248,878, which are both incorporated by reference in their entireties.

[0537] Various modifications to nucleic acid siNA structure can be made to enhance the utility of these molecules. Such modifications will enhance shelf-life, half-life in vitro, stability, and ease of introduction of such oligonucleotides to the target site, e.g., to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

[0538] By "cholesterol derivative" is meant, any compound consisting essentially of a cholesterol structure, including additions, substitutions and/or deletions thereof. The term cholesterol derivative herein also includes steroid hormones and bile acids as are generally recognized in the art.

Administration of Formulated siNA Compositions

[0539] A formulated molecular composition of the invention can be adapted for use to prevent, inhibit, or reduce any trait, disease or condition that is related to or will respond to the levels of target gene expression in a cell or tissue, alone or in combination with other therapies.

[0540] In one embodiment, formulated molecular compositions can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, by injection, by iontophoresis or by incorporation into other vehicles, such as biodegradable polymers, hydrogels, cyclodextrins (see for example Gonzalez et al., 1999, *Bioconjugate Chem.*, 10, 1068-1074; Wang et al., International PCT publication Nos. WO 03/47518 and WO 03/46185). In one embodiment, a formulated molecular compositions of the invention are complexed with membrane disruptive agents such as those described in U.S. Patent Application Publication No. 20010007666, incorporated by reference herein in its entirety including the drawings. In another embodiment, the membrane disruptive agent or agents and the biologically active molecule are also complexed with a cationic lipid or helper lipid molecule, such as those lipids described in U.S. Pat. No. 6,235,310, incorporated by reference herein in its entirety including the drawings.

[0541] In one embodiment, delivery systems of the invention include, for example, aqueous and nonaqueous gels, creams, multiple emulsions, microemulsions, ointments, aqueous and nonaqueous solutions, lotions, aerosols, hydrocarbon bases and powders, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (e.g., polycarbophil and polyvinylpyrolidone). In one embodiment, the pharmaceutically acceptable carrier is a transdermal enhancer.

[0542] In one embodiment, delivery systems of the invention include patches, tablets, suppositories, pessaries, gels and creams, and can contain excipients such as solubilizers and enhancers (e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

[0543] In one embodiment, the invention features a pharmaceutical composition comprising one or more formulated siNA compositions of the invention in an acceptable carrier, such as a stabilizer, buffer, and the like. The formulated molecular compositions of the invention can be administered and introduced to a subject by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. The compositions of the present invention can also be formulated and used as creams, gels, sprays, oils and other suitable compositions for topical, dermal, or transdermal administration as is known in the art.

[0544] In one embodiment, the invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, e.g., acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

[0545] A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, e.g., systemic or local administration, into a cell or subject, including for example a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (i.e., a cell to which the siNA is desirable for delivery). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms that prevent the composition or formulation from exerting its effect.

[0546] In one embodiment, formulated molecular compositions of the invention are administered to a subject by systemic administration in a pharmaceutically acceptable composition or formulation. By "systemic administration" is meant in vivo systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes that lead to systemic absorption include, without limitation: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes exposes the siNA molecules of the invention to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size.

[0547] By "pharmaceutically acceptable formulation" or "pharmaceutically acceptable composition" is meant, a com-

position or formulation that allows for the effective distribution of the formulated molecular A compositions of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the formulated molecular compositions of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85); biodegradable polymers, such as poly(DL-lactide-coglycolide) microspheres for sustained release delivery (Emerich, D F et al, 1999, *Cell Transplant*, 8, 47-58); and loaded nanoparticles, such as those made of polybutylcyanoacrylate. Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado et al., 1998, *J. Pharm. Sci.*, 87, 1308-1315; Tyler et al., 1999, *FEBS Lett.*, 421, 280-284; Pardridge et al., 1995, *PNAS USA.*, 92, 5592-5596; Boado, 1995, *Adv. Drug Delivery Rev.*, 15, 73-107; Aldrian-Herrada et al., 1998, *Nucleic Acids Res.*, 26, 4910-4916; and Tyler et al., 1999, *PNAS USA.*, 96, 7053-7058.

[0548] The present invention also includes compositions prepared for storage or administration that include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A. R. Gennaro edit. 1985), hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

[0549] A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the formulated siNA composition.

[0550] The formulated molecular compositions of the invention can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (e.g., intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a formulated molecular composition of the invention and a pharmaceutically acceptable carrier. One or more formulated molecular compositions of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing formulated molecular compositions of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

[0551] Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents; such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. In some cases such coatings can be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate can be employed.

[0552] Formulations for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

[0553] Aqueous suspensions contain the active materials in a mixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

[0554] Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid.

[0555] Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or

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more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

[0556] Pharmaceutical compositions of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

[0557] Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

[0558] The formulated molecular compositions of the invention can also be administered in the form of suppositories, e.g., for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

[0559] Formulated molecular compositions of the invention can be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

[0560] Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per subject per day). The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Dosage unit forms generally contain between from about 1 mg to about 500 mg of an active ingredient.

[0561] It is understood that the specific dose level for any particular subject depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administra-

tion, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

[0562] For administration to non-human animals, the composition can also be added to the animal feed or drinking water. It can be convenient to formulate the animal feed and drinking water compositions so that the animal takes in a therapeutically appropriate quantity of the composition along with its diet. It can also be convenient to present the composition as a premix for addition to the feed or drinking water.

[0563] The formulated molecular compositions of the present invention can also be administered to a subject in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication can increase the beneficial effects while reducing the presence of side effects.

EXAMPLES

[0564] The following are non-limiting examples showing the selection, isolation, synthesis and activity of nucleic acids of the instant invention.

Example 1

Identification of Potential siNA Target Sites in any RNA Sequence

[0565] The sequence of an RNA target of interest, such as a viral or human mRNA transcript, is screened for target sites, for example by using a computer folding algorithm. In a non-limiting example, the sequence of a gene or RNA gene transcript derived from a database, such as Genbank, is used to generate siNA targets having complementarity to the target. Such sequences can be obtained from a database, or can be determined experimentally as known in the art. Target sites that are known, for example, those target sites determined to be effective target sites based on studies with other nucleic acid molecules, for example ribozymes or antisense, or those targets known to be associated with a disease or condition such as those sites containing mutations or deletions, can be used to design siNA molecules targeting those sites. Various parameters can be used to determine which sites are the most suitable target sites within the target RNA sequence. These parameters include but are not limited to secondary or tertiary RNA structure, the nucleotide base composition of the target sequence, the degree of homology between various regions of the target sequence, or the relative position of the target sequence within the RNA transcript. Based on these determinations, any number of target sites within the RNA transcript can be chosen to screen siNA molecules for efficacy, for example by using in vitro RNA cleavage assays, cell culture, or animal models. In a non-limiting example, anywhere from 1 to 1000 target sites are chosen within the transcript based on the size of the siNA construct to be used. High throughput screening assays can be developed for screening siNA molecules using methods known in the art, such as with multi-well or multi-plate assays to determine efficient reduction in target gene expression. These methods can also be used to determine target sites for, example, antisense, ribozyme, 2-5-A, triplex, and decoy nucleic acid molecules of the invention.

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Example 2

Selection of siNA Molecule Target Sites in a RNA

[0566]   The following non-limiting steps can be used to carry out the selection of siNAs targeting a given gene sequence or transcript.

[0567]   1. The target sequence is parsed in silico into a list of all fragments or subsequences of a particular length, for example 23 nucleotide fragments, contained within the target sequence. This step is typically carried out using a custom Perl script, but commercial sequence analysis programs such as Oligo, MacVector, or the GCG Wisconsin Package can be employed as well.

[0568]   2. In some instances the siNAs correspond to more than one target sequence; such would be the case for example in targeting different transcripts of the same gene, targeting different transcripts of more than one gene, or for targeting both the human gene and an animal homolog. In this case, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find matching sequences in each list. The subsequences are then ranked according to the number of target sequences that contain the given subsequence; the goal is to find subsequences that are present in most or all of the target sequences. Alternately, the ranking can identify subsequences that are unique to a target sequence, such as a mutant target sequence. Such an approach would enable the use of siNA to target specifically the mutant sequence and not effect the expression of the normal sequence.

[0569]   3. In some instances the siNA subsequences are absent in one or more sequences while present in the desired target sequence; such would be the case if the siNA targets a gene with a paralogous family member that is to remain untargeted. As in case 2 above, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find sequences that are present in the target gene but are absent in the untargeted paralog.

[0570]   4. The ranked siNA subsequences can be further analyzed and ranked according to GC content. A preference can be given to sites containing 30-70% GC, with a further preference to sites containing 40-60% GC.

[0571]   5. The ranked siNA subsequences can be further analyzed and ranked according to self-folding and internal hairpins. Weaker internal folds are preferred; strong hairpin structures are to be avoided.

[0572]   6. The ranked siNA subsequences can be further analyzed and ranked according to whether they have runs of GGG or CCC in the sequence. GGG (or even more Gs) in either strand can make oligonucleotide synthesis problematic and can potentially interfere with RNAi activity, so it is avoided whenever better sequences are available. CCC is searched in the target strand because that will place GGG in the antisense strand.

[0573]   7. The ranked siNA subsequences can be further analyzed and ranked according to whether they have the dinucleotide UU (uridine dinucleotide) on the 3'-end of the sequence, and/or AA on the 5'-end of the sequence (to

yield 3' UU on the antisense sequence). These sequences allow one to design siNA molecules with terminal TT thymidine dinucleotides.

[0574]   8. Four or five target sites are chosen from the ranked list of subsequences as described above. For example, in subsequences having 23 nucleotides, the right 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the upper (sense) strand of the siNA duplex, while the reverse complement of the left 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the lower (antisense) strand of the siNA duplex. If terminal TT residues are desired for the sequence (as described in paragraph 7), then the two 3' terminal nucleotides of both the sense and antisense strands are replaced by TT prior to synthesizing the oligos.

[0575]   9. The siNA molecules are screened in an in vitro, cell culture or animal model system to identify the most active siNA molecule or the most preferred target site within the target RNA sequence.

[0576]   10. Other design considerations can be used when selecting target nucleic acid sequences, see, for example, Reynolds et al., 2004, *Nature Biotechnology Advanced Online Publication*, 1 Feb. 2004, doi:10.1038/nbt936 and Ui-Tei et al., 2004, Nucleic Acids Research, 32, doi:10.1093/nar/gkh247.

Example 3

siNA Design

[0577]   siNA target sites were chosen by analyzing sequences of the target RNA target and optionally prioritizing the target sites on the basis of folding (structure of any given sequence analyzed to determine siNA accessibility to the target), by using a library of siNA molecules, or alternately by using an in vitro siNA system as described herein. siNA molecules are designed that could bind each target and are optionally individually analyzed by computer folding to assess whether the siNA molecule can interact with the target sequence. Varying the length of the siNA molecules can be chosen to optimize activity. Generally, a sufficient number of complementary nucleotide bases are chosen to bind to, or otherwise interact with, the target RNA, but the degree of complementarity can be modulated to accommodate siNA duplexes or varying length or base composition. By using such methodologies, siNA molecules can be designed to target sites within any known RNA sequence, for example those RNA sequences corresponding to the any gene transcript.

[0578]   Chemically modified siNA constructs are designed to provide nuclease stability for systemic administration in vivo and/or improved pharmacokinetic, localization, and delivery properties while preserving the ability to mediate RNAi activity. Chemical modifications as described herein are introduced synthetically using synthetic methods described herein and those generally known in the art. The synthetic siNA constructs are then assayed for nuclease stability in serum and/or cellular/tissue extracts (e.g. liver extracts). The synthetic siNA constructs are also tested in parallel for RNAi activity using an appropriate assay, such as a luciferase reporter assay as described herein or another suitable assay that can quantity RNAi activity. Synthetic

siNA constructs that possess both nuclease stability and RNAi activity can be further modified and re-evaluated in stability and activity assays. The chemical modifications of the stabilized active siNA constructs can then be applied to any siNA sequence targeting any chosen RNA and used, for example, in target screening assays to pick lead siNA compounds for therapeutic development.

Example 4

Chemical Synthesis and Purification of siNA

[0579] siNA molecules can be designed to interact with various sites in the RNA message, for example, target sequences within the RNA sequences described herein. The sequence of one strand of the siNA molecule(s) is complementary to the target site sequences described above. The siNA molecules can be chemically synthesized using methods described herein. Inactive siNA molecules that are used as control sequences can be synthesized by scrambling the sequence of the siNA molecules such that it is not complementary to the target sequence. Generally, siNA constructs can by synthesized using solid phase oligonucleotide synthesis methods as described herein (see for example Usman et al., U.S. Pat. Nos. 5,804,683; 5,831,071; 5,998,203; 6,117,657; 6,353,098; 6,362,323; 6,437,117; 6,469,158; Scaringe et al., U.S. Pat. Nos. 6,111,086; 6,008,400; 6,111,086 all incorporated by reference in their entireties herein).

[0580] In a non-limiting example, RNA oligonucleotides are synthesized in a stepwise fashion using the phosphoramidite chemistry as is known in the art. Standard phosphoramidite chemistry involves the use of nucleosides comprising any of 5'-O-dimethoxytrityl, 2'-O-tert-butyldimethylsilyl, 3'-O-2-Cyanoethyl N,N-diisopropylphos-phoroamidite groups, and exocyclic amine protecting groups (e.g. N6-benzoyl adenosine, N4 acetyl cytidine, and N2-isobutyryl guanosine). Alternately, 2'-O-Silyl Ethers can be used in conjunction with acid-labile 2'-O-orthoester protecting groups in the synthesis of RNA as described by Scaringe supra. Differing 2' chemistries can require different protecting groups, for example 2'-deoxy-2'-amino nucleosides can utilize N-phthaloyl protection as described by Usman et al., U.S. Pat. No. 5,631,360, incorporated by reference herein in its entirety).

[0581] During solid phase synthesis, each nucleotide is added sequentially (3'- to 5'-direction) to the solid support-bound oligonucleotide. The first nucleoside at the 3'-end of the chain is covalently attached to a solid support (e.g., controlled pore glass or polystyrene) using various linkers. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are combined resulting in the coupling of the second nucleoside phosphoramidite onto the 5'-end of the first nucleoside. The support is then washed and any unreacted 5'-hydroxyl groups are capped with a capping reagent such as acetic anhydride to yield inactive 5'-acetyl moieties. The trivalent phosphorus linkage is then oxidized to a more stable phosphate linkage. At the end of the nucleotide addition cycle, the 5'-O-protecting group is cleaved under suitable conditions (e.g., acidic conditions for trityl-based groups and Fluoride for silyl-based groups). The cycle is repeated for each subsequent nucleotide.

[0582] Modification of synthesis conditions can be used to optimize coupling efficiency, for example by using differing coupling times, differing reagent/phosphoramidite concentrations, differing contact times, differing solid supports and solid support linker chemistries depending on the particular chemical composition of the siNA to be synthesized. Deprotection and purification of the siNA can be performed as is generally described in Usman et al., U.S. Pat. No. 5,831,071, U.S. Pat. No. 6,353,098, U.S. Pat. No. 6,437,117, and Bellon et al., U.S. Pat. No. 6,054,576, U.S. Pat. No. 6,162,909, U.S. Pat. No. 6,303,773, or Scaringe supra, incorporated by reference herein in their entireties. Additionally, deprotection conditions can be modified to provide the best possible yield and purity of siNA constructs. For example, applicant has observed that oligonucleotides comprising 2'-deoxy-2'-fluoro nucleotides can degrade under inappropriate deprotection conditions. Such oligonucleotides are deprotected using aqueous methylamine at about 35° C. for 30 minutes. If the 2'-deoxy-2'-fluoro containing oligonucleotide also comprises ribonucleotides, after deprotection with aqueous methylamine at about 35° C. for 30 minutes, TEA-HF is added and the reaction maintained at about 65° C. for an additional 15 minutes. siNA molecules that are deprotected, purified, and/or annealed are then formulated as described herein.

Example 5

RNAi In Vitro Assay to Assess siNA Activity

[0583] An in vitro assay that recapitulates RNAi in a cell-free system is used to evaluate siNA constructs targeting RNA targets. The assay comprises the system described by Tuschl et al., 1999, *Genes and Development*, 13, 3191-3197 and Zamore et al., 2000, *Cell*, 101, 25-33 adapted for use with target RNA. A *Drosophila* extract derived from syncytial blastoderm is used to reconstitute RNAi activity in vitro. Target RNA is generated via in vitro transcription from an appropriate hairless expressing plasmid using T7 RNA polymerase or via chemical synthesis as described herein. Sense and antisense siNA strands (for example 20 uM each) are annealed by incubation in buffer (such as 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 minute at 90° C. followed by 1 hour at 37° C., then diluted in lysis buffer (for example 100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate). Annealing can be monitored by gel electrophoresis on an agarose gel in TBE buffer and stained with ethidium bromide. The *Drosophila* lysate is prepared using zero to two-hour-old embryos from Oregon R flies collected on yeasted molasses agar that are dechorionated and lysed. The lysate is centrifuged and the supernatant isolated. The assay comprises a reaction mixture containing 50% lysate [vol/vol], RNA (10-50 pM final concentration), and 10% [vol/vol] lysis buffer containing siNA (10 nM final concentration). The reaction mixture also contains 10 mM creatine phosphate, 10 ug/ml creatine phosphokinase, 100 um GTP, 100 uM UTP, 100 uM CTP, 500 uM ATP, 5 mM DTT, 0.1 U/uL RNasin (Promega), and 100 uM of each amino acid. The final concentration of potassium acetate is adjusted to 100 mM. The reactions are pre-assembled on ice and preincubated at 25° C. for 10 minutes before adding RNA, then incubated at 25° C. for an additional 60 minutes. Reactions are quenched with 4 volumes of 1.25× Passive Lysis Buffer (Promega). Target RNA cleavage is assayed by RT-PCR analysis or other methods known in the art and are compared to control reactions in which siNA is omitted from the reaction.

[0584] Alternately, internally-labeled target RNA for the assay is prepared by in vitro transcription in the presence of [alpha-^{32}P] CTP, passed over a G 50 Sephadex column by spin chromatography and used as target RNA without further purification. Optionally, target RNA is 5'-^{32}P-end labeled using T4 polynucleotide kinase enzyme. Assays are performed as described above and target RNA and the specific RNA cleavage products generated by RNAi are visualized on an autoradiograph of a gel. The percentage of cleavage is determined by PHOSPHOR IMAGER® (autoradiography) quantitation of bands representing intact control RNA or RNA from control reactions without siNA and the cleavage products generated by the assay.

[0585] In one embodiment, this assay is used to determine target sites the RNA target for siNA mediated RNAi cleavage, wherein a plurality of siNA constructs are screened for RNAi mediated cleavage of the RNA target, for example, by analyzing the assay reaction by electrophoresis of labeled target RNA, or by northern blotting, as well as by other methodology well known in the art.

Example 6

Nucleic Acid Inhibition of Target RNA

[0586] siNA molecules targeted to the human target RNA are designed and synthesized as described above. These nucleic acid molecules can be tested for cleavage activity in vivo, for example, using the following procedure.

[0587] Two formats are used to test the efficacy of siNAs targeting target. First, the reagents are tested in cell culture to determine the extent of RNA and protein inhibition. siNA reagents are selected against the target as described herein. RNA inhibition is measured after delivery of these reagents by a suitable transfection agent to cells. Relative amounts of target RNA are measured versus actin using real-time PCR monitoring of amplification (e.g., ABI 7700 TAQMAN®). A comparison is made to a mixture of oligonucleotide sequences made to unrelated targets or to a randomized siNA control with the same overall length and chemistry, but randomly substituted at each position. Primary and secondary lead reagents are chosen for the target and optimization performed. After an optimal transfection agent concentration is chosen, a RNA time-course of inhibition is performed with the lead siNA molecule. In addition, a cell-plating format can be used to determine RNA inhibition.

Delivery of siNA to Cells

[0588] Cells are seeded, for example, at 1×10^5 cells per well of a six-well dish in EGM-2 (BioWhittaker) the day before transfection. Formulated siNA compositions are complexed in EGM basal media (Bio Whittaker) at 37° C. for 30 minutes in polystyrene tubes. Following vortexing, the complexed formulated siNA composition is added to each well and incubated for the times indicated. For initial optimization experiments, cells are seeded, for example, at 1×10^3 in 96 well plates and siNA complex added as described. Efficiency of delivery of siNA to cells is determined using a fluorescent siNA complexed with lipid. Cells in 6-well dishes are incubated with siNA for 24 hours, rinsed with PBS and fixed in 2% paraformaldehyde for 15 minutes at room temperature. Uptake of siNA is visualized using a fluorescent microscope.

TAQMAN® (Real-Time PCR Monitoring of Amplification) and Lightcycler Quantification of mRNA

[0589] Total RNA is prepared from cells following siNA delivery, for example, using Qiagen RNA purification kits for 6-well or Rneasy extraction kits for 96-well assays. For TAQMAN® analysis (real-time PCR monitoring of amplification), dual-labeled probes are synthesized with the reporter dye, FAM or JOE, covalently linked at the 5'-end and the quencher dye TAMRA conjugated to the 3'-end. One-step RT-PCR amplifications are performed on, for example, an ABI PRISM 7700 Sequence Detector using 50 μl reactions consisting of 10 μl total RNA, 100 nM forward primer, 900 nM reverse primer, 100 nM probe, 1× TaqMan PCR reaction buffer (PE-Applied Biosystems), 5.5 mM MgCl$_2$, 300 μM each dATP, dCTP, dGTP, and dTTP, 10 U RNase Inhibitor (Promega), 1.25 U AMPLITAQ GOLD® (DNA polymerase) (PE-Applied Biosystems) and 10 U M-MLV Reverse Transcriptase (Promega). The thermal cycling conditions can consist of 30 minutes at 48° C., 10 minutes at 95° C., followed by 40 cycles of 15 seconds at 95° C. and 1 minute at 60° C. Quantitation of mRNA levels is determined relative to standards generated from serially diluted total cellular RNA (300, 100, 33, 11 ng/rxn) and normalizing to β-actin or GAPDH mRNA in parallel TAQMAN® reactions (real-time PCR monitoring of amplification). For each gene of interest an upper and lower primer and a fluorescently labeled probe are designed. Real time incorporation of SYBR Green I dye into a specific PCR product can be measured in glass capillary tubes using a lightcyler. A standard curve is generated for each primer pair using control cRNA. Values are represented as relative expression to GAPDH in each sample.

Western Blotting

[0590] Nuclear extracts can be prepared using a standard micro preparation technique (see for example Andrews and Faller, 1991, *Nucleic Acids Research*, 19, 2499). Protein extracts from supernatants are prepared, for example using TCA precipitation. An equal volume of 20% TCA is added to the cell supernatant, incubated on ice for 1 hour and pelleted by centrifugation for 5 minutes. Pellets are washed in acetone, dried and resuspended in water. Cellular protein extracts are run on a 10% Bis-Tris NuPage (nuclear extracts) or 4-12% Tris-Glycine (supernatant extracts) polyacrylamide gel and transferred onto nitro-cellulose membranes. Non-specific binding can be blocked by incubation, for example, with 5% non-fat milk for 1 hour followed by primary antibody for 16 hour at 4° C. Following washes, the secondary antibody is applied, for example (1:10,000 dilution) for 1 hour at room temperature and the signal detected with SuperSignal reagent (Pierce).

Example 7

Evaluation of Serum Stability of Formulated siNA Compositions

[0591] As discussed herein, one way to determine the transfection or delivery efficiency of the formulated lipid composition is to determine the stability of the formulated composition in serum in vitro. Relative turbity measurement can be used to determine the in vitro serum stability of the formulated siNA compositions.

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[0592]   Turbidity measurements were employed to monitor the serum stability of lipid particle formulations L065, F2, L051, and L073 (see **FIGS. 8 and 9** for the lipid formulations of L051 and L073). The lipid formulation of L065 comprises cationic lipid CpLinDMA, neutral lipid DSPC, cholesterol, and 2kPEG-DMG. The lipid formulation F2 comprises DODAP. The absorbance of formulated siNA compositions (0.1 mg/ml) in the absence and presence of 50% serum was measured at 500 nm with a corresponding amount of serum alone as a reference by using SpectraMax® Plus384 microplate spectrophotometer from Molecular Devices (Sunnyvale, Calif.). The formulations were incubated at 37° C. and analyzed at 2 min, 5 min, 10 min, 20 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 7 h and 24 h. Relative turbidity was determined by dividing the sample turbidity by the turbidity of 2 min formulated siNA compositions incubated in 50% serum. A formulated molecular composition is stable in serum if the relative turbidity remains constant around 1.0 over time. As shown in **FIG. 11**, formulated siNA compositions L065, L051, and L073 are serum-stable lipid nanoparticle compositions. As shown in **FIG. 33**, formulated siNA compositions L077, L080, L082 and L083, are serum-stable lipid nanoparticle compositions.

Example 8

Evaluation of pH-Dependent Phase Transition of Formulated siNA Compositions

[0593]   Additionally, the transfection or delivery efficiency of the formulated lipid composition can be determined by determining the pH-dependent phase transition of the formulated composition in vitro. Relative turbity measurement can be used to determine the pH-dependent phase transition of formulated siNA compositions in vitro.

[0594]   Turbidity measurement was employed to monitor the phase transition of formulated siNA compositions L065, L051, F2, L073, and L069. The absorbance of lipid particle formulations (0.1 mg/ml) in 0.1 M phosphate buffer with pH at 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 was measured at 350 nm with a corresponding amount of buffer alone as a reference by using SpectraMax® Plus384 microplate spectrophotometer from Molecular Devices (Sunnyvale, Calif.). This assay measures the relative light scattering of the formulations at various pH. The lamellar structure (i.e., serum stable structure) having realatively bigger particle size is expected to scatter more light than the corresponding inverted hexagonal structure. The samples were incubated at 37° C. and analyzed at 2 min, 5 min, 10 min, 30 min, and 2 h. Relative turbidity was determined by dividing the sample turbidity by the turbidity of 2 min formulated siNA compositions incubated in phosphate buffer at pH 7.5. A formulated molecular composition undergoes pH-dependent phase transition if there is a change in the relative turbidity when measured between pH 7.5-pH 5.0. As shown in **FIG. 12**, formulated siNA compositions L051 and L073 undergo pH-dependent phase transition at pH 6.5-pH 5.0. As shown in **FIG. 13**, formulated siNA composition L069 undergoes pH-dependent phase transition at pH 6.5-pH 5.0. As shown in **FIG. 34**, formulated siNA compositions L077, L080, L082, and L083 undergo pH-dependent phase transition at pH 6.5-pH 5.0.

Example 9

Evaluation of Formulated siNA Compositions in Models of Chronic HBV Infection

In Vitro Analysis of siNA Nanoparticle Activity

[0595]   Hep G2 cells were grown in EMEM (Cellgro Cat# 10-010-CV) with non-essential amino acids, sodium pyruvate (90%), and 10% fetal bovine serum (HyClone Cat#SH30070.03). Replication competent cDNA was generated by the excision and re-ligation of the HBV genomic sequences from the psHBV-1 vector. HepG2 cells were plated (3×10^4 cells/well) in 96-well microtiter plates and incubated overnight. A cationic lipid/DNA complex was formed containing (at final concentrations) cationic lipid (11-15 µg/mL), and re-ligated psHBV-1 (4.5 µg/mL) in growth media. Following a 15 min incubation at 37° C., 20 µL of the complex was added to the plated HepG2 cells in 80 µL of growth media minus antibiotics. After 7.5 hours at 37° C., the media was then removed, the cells rinsed once with media, and 100 µL of fresh media was added to each well. 50 µL of the siNA nanoparticle formulation (see Example 9 for formulation details) (diluted into media at a 3× concentration) was added per well, with 3 replicate wells per concentration. The cells were incubated for 4 days, the media was then removed, and assayed for HBsAg levels. **FIG. 15** shows level of HBsAg from formulated (Formulation L051, Table IV) active siNA treated cells compared to untreated or negative control treated cells. **FIG. 16** shows level of HBsAg from formulated (Formulations L053 and L054, Table IV) active siNA treated cells compared to untreated or negative control treated cells. **FIG. 17** shows level of HBsAg from formulated (Formulation L051, Table IV) active siNA treated cells compared to untreated or negative control treated cells. **FIG. 30** shows level of HBsAg from formulated (Formulations L083 and L084, Table IV) active siNA treated cells compared to untreated or negative control treated cells. **FIG. 31** shows level of HBsAg from formulated (Formulation L077, Table IV) active siNA treated cells compared to untreated or negative control treated cells. **FIG. 32** shows level of HBsAg from formulated (Formulation L080, Table IV) active siNA treated cells compared to untreated or negative control treated cells. In these studies, a dose dependent reduction in HBsAg levels was observed in the active formulated siNA treated cells using nanoparticle formulations L051, L053, and L054, while no reduction is observed in the negative control treated cells. This result indicates that the formulated siNA compositions are able to enter the cells, and effectively engage the cellular RNAi machinery to inhibit viral gene expression.

Analysis of Formulated siRNA Activity in a Mouse Model of HBV Replication

[0596]   To assess the activity of chemically stabilized siNA nanoparticle (see Example 9 for formulation details) compositions against HBV, systemic dosing of the formulated siNA composition (Formulation L051, Table IV) was performed following hydrodynamic injection (HDI) of HBV vector in mouse strain NOD.CB17-Prkdc^{scid}/J (Jackson Laboratory, Bar Harbor, Me.). Female mice were 5-6 weeks of age and approximately 20 grams at the time of the study. The HBV vector used, pWTD, is a head-to-tail dimer of the complete HBV genome. For a 20-gram mouse, a total

injection of 1.6 ml containing pWTD in saline, was injected into the tail vein within 5 seconds. A total of 0.3 µg of the HBV vector was injected per mouse. In order to allow recovery of the liver from the disruption caused by HDI, dosing of the formulated siNA compositions were started 6 days post-HDI. Encapsulated active or negative control siRNA were administered at 3 mg/kg/day for three days via standard IV injection. Groups (N=5) of animals were sacrificed at 3 and 7 days following the last dose, and the levels of serum HBV DNA and HBsAg were measured. HBV DNA titers were determined by quantitative real-time PCR and expressed as mean log10 copies/ml (±SEM). The serum HBsAg levels were assayed by ELISA and expressed as mean log10 pg/ml (±SEM). Significant reductions in serum HBV DNA (**FIGS. 18 and 29**) and HBsAg (**FIGS. 19, 30, 31, and 32**)) were observed at both the 3 and 7-day time points in the active formulated siNA composition treated groups as compared to both the PBS and negative control groups.

Materials and Methods

Oligonucleotide Synthesis and Characterization

[0597] All RNAs were synthesized as described herein. Complementary strands were annealed in PBS, desalted and lyophilized. The sequences of the active site 263 HBV siNAs are shown in **FIG. 14**. The modified siNAs used in vivo are termed HBV263M and HBV1583M, while versions containing unmodified ribonucleotides and inverted abasic terminal caps are called HBV263R and HBV1583R. Some pharmacokinetic studies were done with siNA targeting two other sites, HBV1580M and HBV1580R.

[0598] The siNA sequences for HCV irrelevant control are:

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sense strand:
5' B-cuGAuAGGGuGcuuGcGAGTT-B 3'    (SEQ ID NO:1)

antisense strand:
5' CUCGcAAGcAcccuAucAGTsT 3'       (SEQ ID NO:2)
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[0599] (where lower case=2'-deoxy-2'-flouro; Upper Case italic=2'-deoxy; Upper Case underline=2'-O-methyl; Upper Case Bold=ribonucleotide; T=thymidine; B=inverted deoxyabasic; and s=phosphorothioate)

[0600] The inverted control sequences are inverted from 5' to 3'.

HBsAg ELISA Assay

[0601] Levels of HBsAg were determined using the Genetic Systems/Bio-Rad (Richmond, Va.) HBsAg ELISA kit, as per the manufacturer's instructions. The absorbance of cells not transfected with the HBV vector was used as background for the assay, and thus subtracted from the experimental sample values.

HBV DNA Analysis

[0602] Viral DNA was extracted from 50 µL mouse serum using QIAmp 96 DNA Blood kit (Qiagen, Valencia, Calif.), according to manufacture's instructions. HBV DNA levels were analyzed using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, Calif.). Quantitative real time PCR was carried out using the following primer and probe sequences: forward primer 5'-CCTGTATTCCCATC-

CCATCGT (SEQ ID NO: 3, HBV nucleotide 2006-2026), reverse primer 5'-TGAGCCAAGAGAAACGGACTG (SEQ ID NO: 4, HBV nucleotide 2063-2083) and probe FAM 5'-TTCGCAAAATACCTATGGGAGTGGGCC (SEQ ID NO: 5, HBV nucleotide 2035-2062). The psHBV-1 vector, containing the full length HBV genome, was used as a standard curve to calculate HBV copies per mL of serum.

Example 10

Evaluation of Formulated siNA Compositions in an In Vitro HCV Replicon Model of HCV Infection

[0603] An HCV replicon system was used to test the efficacy of siNAs targeting HCV RNA. The reagents were tested in cell culture using Huh7 cells (see for example Randall et al., 2003, PNAS USA, 100, 235-240) to determine the extent of RNA inhbition. siNA were selected against the HCV target as described herein. The active siNA sequences for HCV site 304 are as follows: sense strand: (SEQ ID NO: 1); antisense strand: (SEQ ID NO: 2) (these were used as inactive sequences in Example 8 above). The siNA inactive control sequences used in the study target HBV site 263 and are as follows: sense strand: (SEQ ID NO: 6); antisense strand: (SEQ ID NO: 7), (these were used as active sequences in Example 8 above). The active and inactive siNAs were formulated as Formulation L051, L053, or L054 as described in Example 9 above. Huh7 cells, containing the stably transfected Clone A HCV subgenomic replicon (Apath, LLC, St. Louis, Mo.), were grown in DMEM (Invitrogen catalog # 11965-118) with 5 mls of 100× (10 mM) Non-Essential Amino Acids (Invitrogen catalog #11140-050), 5 uL of 200 mM Glutamine (Cellgro catalog#25-005-C1), 50 uL of heat inactivated Fetal Bovine Serum (Invitrogen catalog #26140-079) and 1 mg/mLG418 (Invitrogen catalog#11811-023). For transfection with siNA formuations, cells are plated at 9,800 cells per well into a 96-well CoStar tissue culture plate using DMEM with NEAA and 10% FBS, (no antibiotics). After 20-24 hours, cells were transfected with formulated siNA for a final concentration of 1-25 nM. After incubating for 3 days, the cells were lysed and RNA extracted using the RNaqueous-96 kit (Ambion Cat#1920) as per the manufacturers instructions. **FIG. 20** shows level of HCV RNA from formulated (Formulation L051, Table IV) active siNA treated cells compared to untreated or negative control treated cells. **FIG. 21** shows level of HCV RNA from formulated (Formulations L053 and L054, Table IV) active siNA treated cells compared to untreated or negative control treated cells. In these studies, a dose dependent reduction in HCV RNA levels was observed in the active formulated siNA treated cells using formulations L051, L053, and L054, while no reduction is observed in the negative control treated cells. This result indicates that the formulated siNA compositions are able to enter the cells, and effectively inhibit viral gene expression.

Example 11

Lung Distribution of Unformulated and Formulated siNA After Intratracheal Dosing

[0604] To determine the efficiency of delivery of siNA molecules to the lung, unformulated siRNA (naked), cholesterol conjugated siNA, or siRNA in formulated molecular compositions (T018.1 and T019.1) were administered via

the trachea to the lungs of mice. Unformulated siNA comprises naked nucleic acid. Cholesterol conjugated siNA comprises siNA linked to cholesterol. Formulated molecular compositions T018.1 and T019.1 comprise siNA formulated with DOcarbDAP, DSPC, cholesterol and PEG-DMG, and DODMA, DSPC, cholesterol and PEG-DMG, respectively. Groups of three female C57 Bl/6 mice were placed under anesthesia with ketamine and xylazine. Filtered dosing solutions were administered via the trachea at 1.0 mg/kg duplexed siRNA, using a Penncentury model #1A-1C microsprayer and a Penncentury model #FMJ250 syringe to aerosolize the siRNA (TGFβ site 1264 stabilization chemistry 7/8) directly into the lungs. Animals were dosed with unformulated siNA, cholesterol-conjugated siNA or siNA in formulated molecular compositions. At 1, 24 or 72 hours after dosing, the animals were euthanized, exsanguinated and perfused with sterile veterinary grade saline via the heart. The lungs were removed, placed in a pre-weighed homogenization tube and frozen on dry ice. Lung weights were determined by subtraction after weighing the tubes plus lungs. Levels of siNA in the lung tissue were determined using a hybridization assay. **FIG. 22**, shows the levels of siNA in lung tissue after direct dosing of (i) unformulated siNA, (ii) cholesterol conjugated siNA or (iii) siNA in formulated molecular compositions T018.1 or T019.1. Half lives of exposure in lung tissue were 3-4 hours for the unformulated siNA, 9 hours for the cholesterol conjugated siNA and 37-39 hours for the siNA in formulated molecular compositions T018.1 or T019.1.

Example 12

Efficient Transfection of Various Cell Lines Using siNA LNP Formulations of the Invention

[0605] The transfection efficacy of LNP formuations of the invention was determined in various cell lines, including 6.12 spleen, Raw 264.7 tumor, MM14Lu, NIH 3T3, D10.G4.1 Th2 helper, and lung primary macrophage cells by targeting endogenous MAP Kinase 14 (p38) gene expression. A potent lead siNA against MapK14 (p38a) was selected by in vitro screening using Lipofectamine 2000 (LF2K) as the delivery agent. The sense strand sequence of this siNA comprised 5'-B cuGGuAcAGAccAuAuuGATT B-3' (SEQ ID NO: 6) and the antisense strand sequence comprised 5'-UCAAuAuGGucuGuAccAGTsT-3' (SEQ ID NO: 7), where lower case=2'-deoxy-2'-flouro; Upper Case italic=2'-deoxy; Upper Case underline=2'-O-methyl; Upper Case Bold=ribonucleotide; T=thymidine; B=inverted deoxyabasic; and s=phosphorothioate).

[0606] Proprietary MapK14 targeted LNPs were screened and compared to LF2K and a LNP control containing an inactive siNA in cultured cells. Furthermore, lead LNPs were tested in a dose response method to determine IC50 values. Results are summarized in Table V. **FIG. 35** shows efficacy data for LNP 58 and LNP 98 formulations targeting MapK14 site 1033 in RAW 264.7 mouse macrophage cells. **FIG. 36** shows efficacy data for LNP 98 formulations targeting MapK14 site 1033 in MM14.Lu normal mouse lung cells. **FIG. 37** shows efficacy data for LNP 54, LNP 97, and LNP 98 formulations targeting MapK14 site 1033 in 6.12 B lymphocyte cells. **FIG. 38** shows efficacy data for LNP 98 formulations targeting MapK14 site 1033 in NIH 3T3 cells. **FIG. 39** shows the dose-dependent reduction of

MapK14 RNA via MapK14 LNP 54 and LNP 98 formulated siNAs in RAW 264.7 cells. **FIG. 40** shows the dose-dependent reduction of MapK14 RNA via MapK14 LNP 98 formulated siNAs in MM14.Lu cells. **FIG. 41** shows the dose-dependent reduction of MapK14 RNA via MapK14 LNP 97 and LNP 98 formulated siNAs in 6.12 B cells. **FIG. 42** shows the dose-dependent reduction of MapK14 RNA via MapK14 LNP 98 formulated siNAs in NIH 3T3 cells.

LF2K Transfection Method:

[0607] The following procecure was used for LF2K transfection. After 20-24 hours, cells were transfected using 0.25 or 0.35 uL Lipofectamine 2000/well and 0.15 or 0.25 uL/well, complexed with 25 nM siNA. Lipofectamine 2000 was mixed with OptiMEM and allowed to sit for at least 5 minutes. For 0.25 uL transfections, 1 uL of LF2K was mixed with 99 uL OptiMEM for each complex. For 0.35 uL transfections, 1.4 uL of LF2K was mixed with 98.6 uL OptiMEM for each complex. For 0.15 uL transfections, 0.60 uL of SilentFect was mixed with 99.4 uL OptiMEM for each complex. For 0.30 uL transfections, 1.2 uL of SilentFect was mixed with 98.2 uL OptiMEM for each complex. The siNA was added to a microtitre tube (BioRad #223-9395) and OptiMEM was then added to make 100 uL total volume to be used in 4 wells. 100 uL of the Lipofectamine 2000/OptiMEM mixture was added and the tube was vortexed on medium speed for 10 seconds and allowed to sit at room temperature for 20 minutes. The tube was vortexed quickly and 50 uL was added per well, which contained 100 uL media. RNA from treated cells was isolated at 24, 48, 72, and 96 hours.

LNP Transfection Method:

[0608] The following procecure was used for LF2K transfection. Cells were plated to the desired concentration in 100 uL of complete growth medium in 96-well plates, ranging from from 5,000-30,000 cells/well. After 24 hours, the cells were transfected by diluting a 5× concentration of LNP in complete growth medium onto the cells, (25 uL of 5× results in a final concentration of 1×). RNA from treated cells was isolated at 24, 48, 72, and 96 hours.

Example 13

Reduction of Airway Hyper-Responsiveness in a Mouse Model of Asthma

[0609] An OVA induced airway hyper-responsiveness model was used to evaluate LNP formulated siNA molecules targeting interleukin 4R (IL-4R alpha) for efficacy in reducing airway hyper-responsiveness. The sense strand sequence of the active siNA targeting IL-4R alpha used in this study comprised 5'-B ucAGcAuuAccAAGAuuAATT B-3' (SEQ ID NO: 8) and the antisense strand sequence comprised 5'-UUAAucuuGGuAAuGcuGATsT-3' (SEQ ID NO: 9), where lower case=2'-deoxy-2'-flouro; Upper Case italic=2'-deoxy; Upper Case underline=2'-O-methyl; Upper Case Bold=ribonucleotide; T=thymidine; B=inverted deoxyabasic; and s=phosphorothioate). On Day 0 and 7, the animals were immunized by intraperitoneal injection of 0.4 mg/mL OVA/saline solution mixed in an equal volume of Inject Alum for a final injection solution of 0.2 mg/mL (100 uL/mouse). LNP-51 formulated IL-4R-alpha Site 1111 siNA (see U.S. Ser. No. 11/001,347, incorporated by reference herein), prepared in PBS (w/o Ca2+, Mg2+), or irrelevant

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control was delivered by intratracheal dosing qd (once every day) beginning on Day 17 and ending on Day 26 for a total of 10 doses. Mice were aerosol challenged with OVA (1.5% in saline) for 30 minutes on days 24, 25 and 26 using the Pari LC aerosol nebulizer. Animals were allowed to rest for 24 hours prior to airway function analysis. On Day 28 airway responsiveness was assessed after challenge with aerosolized methacholine using the Buxco Whole Body Plethysmograph. After methacholine challenge, animals were euthanized. A tracheotomy was performed, and the lungs were lavaged with 0.5 mL of saline twice. Lung lavage was performed while massaging the animal's chest and all lavage fluid were collected and placed on ice. A cytospin preparation was performed to collect the cells from the BAL fluid for differential cell counts. Results are shown in **FIG. 43**, which clearly demonstrates the activity of the formulated siNA in a dose response (0.01, 0.1, and 1 mg/kg) compared to the LNP vehicle alone and untreated (naïve) animals.

Example 14

Efficient Reduction in Human Huntingtin (htt) Gene Expression In Vivo Using LNP Formulated siNA

[0610]   Huntington's disease (HD) is a dominant neurodegenerative disorder caused by an expansion in the polyglutamine (polyQ) tract of the huntingtin (htt) protein. PolyQ expansion in htt induces cortical and striatal neuron cell less, and the formation of htt-containing aggregates within brain cells. HD patients have progressive psychiatric, cognitive and motor dysfunction and premature death. Early work in mouse models has demonstrated that reduction of mutant protein after the onset of disease phenotypes could improve motor dysfunction and reduce htt-aggregate burden. Thus, reduction of mutant htt in patient brain may improve the disease.

[0611]   Recent work has shown that reduction of mutant htt in a mouse model of HD, using a viral vector expressing short interfering RNAs (siRNAs), protected the animal from the onset of behavioral and neuropathological hallmarks of the disease (see Harper et al., 2005, PNAS USA, 102: 5820-5). This study was utilized to determine whether delivery of synthetic siNAs directly to the brain by nonviral methods could be similarly effective. This approach has many advantages, including the ability to modify dosing regimines. Chemically modified siNA, sense strand having sequence 5'-B AccGuGuGAAucAuuGucuTT B-3' (SEQ ID NO:10) and antisense strand 5'-AGAcAAuGAuucAcAcG-GuTsT-3' (SEQ ID NO:11) encapsulated in lipid nanoparticles (LNP) formulations LNP-061, LNP-098, and LNP-101 (see Table IV) were utilized in this study. In these sequences, lower case stands for 2'-deoxy-2'-fluoro, Upper Case stands for ribonucleotides, underline Upper Case stands for 2'-O-methyl nucleotides, T is thymidine, s is phosphorothioate, and B is inverted deoxy abasic. The siNA duplexes encapsulated in the various LNP formulations were screened for their ability to silence full-length htt in vitro, followed by testing in vivo. Using Alzet osmotic pumps, siNAs encapsulated in LNPs were infused into the lateral ventrical or striatum for 7 or 14 days, respectively, at concentrations ranging from 0.1 to 1 mg/ml (total dose ranging from 8.4 to 84 µg). An impressive 80% reduction in htt mRNA levels was observed in animals treated with

LNP-061 and LNP-098 formulated siNA as determined by QPCR compared to scrambled control sequences, or naïve brain. Results are shown in **FIG. 44**.

Example 14

Preparation of Cationic Lipids of the Invention (see Table III for Cationic Lipids and Intermediates See FIG. **23**A for Synthetic Scheme)

Cholest-5-en-3β-tosylate (2)

[0612]   Cholesterol (1, 25.0 g, 64.7 mmol) was weighed into a 1 L round bottomed flask with a stir bar. The flask was charged with pyridine (250 mL), septum sealed and flushed with argon. Toluenesulfonyl chloride (25.0 g, 131 mmol) was weighed into a 100 mL round bottomed flask, which was then sealed and charged with pyridine. The toluenesulfonyl chloride solution was then transferred, via syringe, to the stirring cholesterol solution, which was allowed to stir overnight. The bulk of pyridine was removed in vacuo and the resulting solids were suspended in methanol (300 mL) and stirred for 3 hours, until the solids were broken up into a uniform suspension. The resultant suspension was filtered and the solids were washed with acetonitrile and dried under high vacuum to afford 31.8 g (91%) of a white powder (see for example Davis. S. C.; Szoka, F. C., Jr. Bioconjugate Chem. 1998, 9, 783).

Cholest-5-en-3β-oxybutan-4-ol (3a)

[0613]   Cholest-5-ene-3β-tosylate (20.0 g, 37.0 mmol) was weighed into a 500 mL round bottomed flask with a stir bar. The flask was charged with dioxane (300 mL) and 1,4-butanediol (65.7 mL, 20 equiv.). The flask was fitted with a reflux condenser and the mixture was brought to reflux overnight. The reaction was cooled and concentrated in vacuo. The reaction mixture was suspended in water (400 mL). The solution was extracted with methylene chloride (3×200 mL). The organic phases were combined and washed with water (2×200), dried over magnesium sulfate, filtered and the solvent removed. The resultant oil/wax was further purified via column chromatography (15% Acetone/Hexanes) to afford 13.41 g (79%) of a colorless wax.

Cholest-5-en-3β-oxypent-3-oxa-an-5-ol (3b)

[0614]   This compound was prepared similarly to cholest-5-en-3β-oxybutan-4-ol. Cholest-5-ene-3β-tosylate (5.0 g, 9.2 mmol) was weighed into a 500 mL round bottomed flask with a stir bar. The flask was charged with dioxane (150 mL) and diethylene glycol (22 mL, 25 equiv.). The flask was fitted with a reflux condenser and the mixture was brought to reflux overnight. The reaction was cooled and concentrated. The reaction mixture was suspended in water (500 mL). The solution was extracted with methylene chloride (3×200 mL). The organic phases were combined and washed with water (2×200 mL), dried over magnesium sulfate, filtered and the solvent removed. The resultant oil/wax was further purified via column chromatography (25% EtOAc/Hexanes) to afford 3.60 g (82%) of colorless oil (see for example Davis. S. C.; Szoka, F. C., Jr. Bioconjugate Chem. 1998, 9, 783).

Cholest-5-en-3β-oxybutan-4-mesylate (4a)

[0615]   Cholest-5-en-3β-oxybutan-4-ol (12.45 g, 27.14 mmol) was weighed into a 500 mL round bottomed flask

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with a stir bar. The flask was sealed, flushed with argon, charged with methylene chloride (100 mL) and triethylamine (5.67 mL, 1.5 equiv.) and cooled to 0° C. Methanesulfonyl chloride (3.15 mL, 1.5 equiv.) was measured in a PP syringe and added slowly to the stirring reaction mixture. The reaction was allowed to stir for 1 hr at 0° C. when TLC analysis (7.5% EtOAc/Hexanes) showed that the reaction was complete. The reaction mixture was diluted with methylene chloride (100 mL) and washed with saturated bicarbonate solution (2×200 mL) and brine (1×100 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated to give 14.45 g (99%) of a colorless wax that was used without further purification.

Cholest-5-en-3β-oxypent-3-oxa-an-5-mesylate (4b)

[0616] This compound was prepared similarly to Cholest-5-en-3β-oxybutan-4-mesylate. Cholest-5-en-3β-oxypent-3-oxa-an-5-ol (3.60 g, 7.58 mmol) was weighed into a 500 mL round bottomed flask with a stir bar. The flask was sealed, flushed with argon, charged with methylene chloride (30 mL) and triethylamine (1.60 mL, 1.5 equiv.) and cooled to 0° C. Methanesulfonyl chloride (0.89 mL, 1.5 equiv.) was measured in a PP syringe and added slowly to the stirring reaction mixture. The reaction was allowed to stir for 1 hr at 0° C. when TLC analysis (10% EtOAc/Hexanes) showed that the reaction was complete. The reaction mixture was diluted with methylene chloride (150 mL) and washed with saturated bicarbonate solution (2×100 mL) and brine (1×100 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated to give 4.15 g (99%) of a colorless wax that was used without further purification.

1-(4,4'-Dimethoxytrityloxy)-3-dimethylamino-2-propanol (5)

[0617] 3-Dimethylamino-1,2-propanediol (6.0 g, 50 mmol) was weighed into a 1 L round bottomed flask with a stir bar. The flask was sealed, flushed with argon, charged with pyridine and cooled to 0° C. 4,4'-Dimethoxytrityl chloride (17.9 g, 1.05 equiv.) was weighed into a 100 mL round bottomed flask, sealed and then dissolved in pyridine (80 mL). The 4,4'-dimethoxytrityl chloride solution was transferred to the stirring reaction mixture slowly, using additional fresh pyridine (20 mL) to effect the transfer of residual 4,4'-dimethoxytrityl chloride. The reaction was allowed to come to room temperature while stirring overnight. The reaction was concentrated in vacuo and re-dissolved in dichloromethane (300 mL). The organic phase was washed with saturated bicarbonate (2×200 mL) and brine (1×200 mL), dried over $MgSO_4$, filtered, concentrated and dried under high vacuum to afford 22.19 g of a yellow gum that was used without further purification.

3-Dimethylamino-2-(cholest-5-en-3β-oxybutan-4-oxy)-1-propanol (6a)

[0618] 1-(4,4'-Dimethoxytrityloxy)-3-Dimethylamino-2-propanol (7.50 g, 17.8 mmol) was weighed into a 200 mL round bottomed flask and co-evaporated with anhydrous toluene (2×50 mL). A stir bar was added to the flask which was septum sealed, flushed with argon and charged with toluene (60 mL). Sodium hydride (1.71 g, 4 equiv.) was added at once and the mixture was stirred at room temperature for 20 minutes. Cholest-5-en-3β-oxybutan-4-mesylate was dissolved in anhydrous toluene (20 mL) and added to

the reaction mixture, via syringe. The flask was fitted with a reflux condenser with a continuous argon stream and the reaction was heated to reflux overnight. The reaction mixture was cooled to room temperature in a water bath and ethanol was added dropwise until gas evolution ceased. The reaction mixture was diluted with ethyl acetate (300 mL) and washed with aqueous 10% sodium carbonate (2×300 mL). The aqueous phases were combined and back extracted with ethyl acetate (2×100 mL). The organic phases were combined, dried over $MgSO_4$, filtered and concentrated to an oil in a 500 mL round bottomed flask.

[0619] The flask was fitted with a stir bar, sealed, purged with argon and charged with dichloroacetic acid solution (3% in DCM, 200 mL). Triethylsilane (14.2 mL, 89 mmol) was added to the mixture and the reaction was allowed to stir overnight. The reaction mixture was diluted with DCM (300 mL) and washed with saturated bicarbonate solution (2×200 mL). The aqueous phases were combined and back extracted with DCM (2×100 mL). The organic phases were combined and dried over $MgSO_4$, filtered and concentrated to an oil that was re-dissolved in ethanol (150 mL). Potassium fluoride (10.3 g, 178 mmol) was added to the solution, which was then brought to reflux for 1 hr. The mixture was cooled, concentrated in vacuo, re-dissolved in DCM (200 mL), filtered and concentrated to an oil/crystal mixture. The mixture was re-dissolved in a minimum of DCM and loaded onto a silica gel column which was pre-equilibrated and eluted with 25% EtOAc/Hexanes with 3% TEA to afford 4.89 g (49%) of a colorless wax.

3-Dimethylamino-2-(cholest-5-en-3β-oxypent-3-oxa-an-5-oxy)-1-propanol (6b)

[0620] This compound was prepared similarly to 3-Dimethylamino-2-(Cholest-5-en-3β-oxybutan-4-oxy)-1-propanol. 1-(4,4'-Dimethoxytrityloxy)-3-Dimethylamino-2-propanol (2.65 g, 6.31 mmol) was weighed into a 200 mL round bottomed flask and co-evaporated with anhydrous toluene (2×20 mL). A stir bar was added to the flask which was septum sealed, flushed with argon and charged with toluene (50 mL). Sodium hydride (0.61 g, 4 equiv.) was added at once and the mixture was stirred at room temperature for 20 minutes. Cholest-5-en-3β-oxypent-3-oxa-an-5-mesylate (4.15 g, 7.6 mmol) was dissolved in anhydrous toluene (10 mL) and added to the reaction mixture, via syringe. The flask was fitted with a reflux condenser with a continuous argon stream and the reaction was heated to reflux overnight. The reaction mixture was cooled to room temperature in a water bath and ethanol was added dropwise until gas evolution ceased. The reaction mixture was diluted with ethyl acetate (200 mL) and washed with aqueous 10% sodium carbonate (2×200 mL). The aqueous phases were combined and back extracted with ethyl acetate (2×100 mL). The organic phases were combined, dried over $MgSO_4$, filtered and concentrated to an oil in a 500 mL round bottomed flask.

[0621] The flask was fitted with a stir bar, sealed, purged with argon and charged with dichloroacetic acid solution (3% in DCM, 150 mL). Triethylsilane (4.03 mL, 25.2 mmol) was added to the mixture and the reaction was allowed to stir for 4 hours. The reaction mixture was diluted with DCM (100 mL) and washed with saturated bicarbonate solution (2×200 mL). The aqueous phases were combined and back extracted with DCM (2×100 mL). The organic phases were

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combined and dried over $MgSO_4$, filtered and concentrated to an oil that was re-dissolved in ethanol (100 mL). Potassium fluoride (3.6 g, 63 mmol) was added to the solution, which was then brought to reflux for 1 hr. The mixture was cooled, concentrated in vacuo, re-dissolved in DCM (200 mL), filtered and concentrated to an oil/crystal mixture. The mixture was re-dissolved in a minimum of DCM and loaded onto a silica gel column which was pre-equilibrated and eluted with 25% Acetone/Hexanes with 3% TEA to afford 2.70 g (74%) of a colorless wax.

Linoleyl Mesylate (7)

[0622]   Linoleyl alcohol (10.0 g, 37.5 mmol) was weighed into a 500 mL round bottomed flask with a stir bar. The flask was sealed, flushed with argon, charged with DCM (100 mL) and triethylamine (7.84 mL, 1.5 equiv.) and cooled to 0° C. Methanesulfonyl chloride (4.35 mL), 1.5 equiv.) was measured in a PP syringe and added slowly to the stirring reaction mixture. TLC analysis (7.5% EtOAc/Hexanes) showed the reaction was complete within 1 hr. The reaction was diluted with DCM (100 mL) and washed with saturated bicarbonate solution (2×200 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated to give 12.53 g (97%) of colorless oil that was used without further purification.

3-Dimethylamino-2-(cholest-5-en-3β-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (8a)

[0623]   3-Dimethylamino-2-(Cholest-5-en-3β-oxybutan-4-oxy)-1-propanol (2.6 g, 4.6 mmol) was weighed into a 200 mL round bottomed flask and co-evaporated with anhydrous toluene 2×20 mL). A stir bar was added to the flask, which was then sealed, flushed with argon and charged with anhydrous toluene (100 mL). Sodium hydride (0.7 g, 6 equiv) was added at once and the mixture was stirred, under argon, for 20 minutes. Linoleyl mesylate (4.6 g, 2.3 equiv.) was measured in a PP syringe and added slowly to the reaction mixture. The flask was fitted with a reflux condenser and the apparatus was flushed with argon. The reaction mixture was heated in an oil bath and allowed to stir at reflux overnight. The reaction mixture was then cooled to room temperature in a water bath and ethanol was added dropwise until gas evolution ceased. The reaction mixture was diluted with ethyl acetate (300 mL) and washed with aqueous 10% sodium carbonate (2×200 mL). The aqueous phases were combined and back extracted with ethyl acetate (2×100 mL). The organic phases were combined, dried over $MgSO_4$, filtered and concentrated. The resultant oil was purified via column chromatography (10% EtOAc/Hexanes, 3% TEA) to afford 3.0 g (81%) of a colorless oil.

3-Dimethylamino-2-(cholest-5-en-3β-oxypent-3-oxa-an-5-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA) (8b)

[0624]   This compound was prepared similarly to 3-Dimethylamino-2-(Cholest-5-en-3β-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane.   3-Dimethylamino-2-(Cholest-5-en-3β-oxypent-3-oxa-an-5-oxy)-1-propanol (0.73 g, 1.3 mmol) was weighed into a 100 mL round bottomed flask and co-evaporated with anhydrous toluene. A stir bar was added to the flask, which was then sealed, flushed with argon and charged with anhydrous toluene. Sodium hydride (121 mg, 4 equiv.) was added at once and

the mixture was stirred, under argon, for 20 minutes. Linoleyl mesylate (0.873 g, 2 equiv.) was measured in a PP syringe and added slowly to the reaction mixture. The flask was fitted with a reflux condenser and the apparatus was flushed with argon. The reaction mixture was heated in an oil bath and allowed to stir at reflux overnight. The reaction mixture was then cooled to room temperature in a water bath and ethanol was added dropwise until gas evolution ceased. The reaction mixture was diluted with ethyl acetate (150 mL) and washed with aqueous 10% sodium carbonate (2×100 mL). The aqueous phases were combined and back extracted with ethyl acetate (2×50 mL). The organic phases were combined, dried over Na_2SO_4, filtered and concentrated. The resultant oil was purified via column chromatography (15% EtOAc/Hexanes, 3% TEA) to afford 0.70 g (67%) of colorless oil.

Example 15

Preparation of Aromatic Lipids of the Invention (See FIG. 23B)

Dioleyloxybenzaldehyde, 3a

[0625]   3,4-Dihydroxybenzaldehyde (2.76 g, 20.0 mmol) was weighed into a 200 mL round bottomed flask with a stir bar. The flask was charged with diglyme (100 mL), septum sealed and flushed with argon. Cesium carbonate (19.5 g, 60.0 mmol) was added to the solution slowly in portions. Oleyl mesylate (15.2 g, 44.0 mmol) was added via syringe. The reaction mixture was heated to 100° C. under slight positive pressure of argon. The reaction mixture was cooled to room temperature and filtered. The solids were washed with 1,2-dichloroethane. The combined filtrate and washes were concentrated and then dried under high vacuum at 65° C. to remove residual diglyme. The resultant yellow oil was purified via flash chromatography (5% ethyl acetate in hexanes) to afford 11.4 g (89%) of a yellow oil that turned to yellow wax upon standing at room temperature.

Dilinoleylbenzaldehyde, 3b

[0626]   3,4-Dihydroxybenzaldehyde (2.76 g, 20.0 mmol) was weighed into a 200 mL round bottomed flask with a stir bar. The flask was charged with diglyme (100 mL), septum sealed and flushed with argon. Cesium carbonate (19.5 g, 60.0 mmol) was added to the solution slowly in portions. Linoleyl mesylate (15.2 g, 44.0 mmol) was added via syringe. The reaction mixture was heated to 100° C. under slight positive pressure of argon. The reaction mixture was cooled to room temperature and filtered. The solids were washed with 1,2-dichloroethane. The combined filtrate and washes were concentrated and then dried under high vacuum at 65° C. to remove residual diglyme. The resultant yellow oil was purified via flash chromatography (5% ethyl acetate in hexanes) to afford 11.9 g (94%) of a brown oil.

N,N-Dimethyl-3,4-dioleyloxybenzylamine, 4a

[0627]   To a solution of triethylamineamine (2.0 mL, 14 mmol) in ethanol (20 mL) was added dimethylamine hydrochloride (1.63 g, 20 mmol), titanium tetraisopropoxide (5.96 mL, 20 mmol) and 3,4-dioleyloxybenzaldehyde (6.39 g, 10 mmol). The mixture was allowed to stir under argon for 10 h at room temperature. Sodium borohydride (0.57 g, 15 mmol) was added to the reaction mixture which was then

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allowed to stir at room temperature overnight. Concentrated aqueous ammonia (4 mL) was added slowly to the reaction mixture. The reaction mixture was filtered and the solids washed with dichloromethane. The filtrate was dried over K_2CO_3, filtered and concentrated. The resultant oil was purified via flash chromatography (2-10% acetone in dichloromethane, 0.5% TEA gradient) to afford 5.81 g (87-+%) of a yellow oil.

N,N-Dimethyl-3,4-dilinoleyloxybenzylamine, 4b

[0628]   To a solution of triethylamineamine (2.0 mL, 14 mmol) in ethanol (20 mL) was added dimethylamine hydrochloride (1.63 g, 20 mmol), titanium tetraisopropoxide (5.96 mL, 20 mmol) and 3,4-dilinoleyloxybenzaldehyde (6.35 g, 10 mmol). The mixture was allowed to stir under argon for 10 h at room temperature. Sodium borohydride (0.57 g, 15 mmol) was added to the reaction mixture which was then allowed to stir at room temperature overnight. 6N Aqueous ammonia (30 mL), was added slowly to the reaction mixture followed by dichloromethane. The reaction mixture was filtered. The filtrate was dried over K_2CO_3, filtered and concentrated. The resultant oil was purified via flash chromatography (2-10% acetone in dichloromethane, 0.5% TEA gradient) to afford 4.94 g (74%) of a yellow oil.

Example 16

Preparation of PEG-Conjugates of the Invention (See FIG. 24)

1-[8'-(Cholest-5-en-3β-oxy)carboxamido-3',6'-dioxaoctanyl]carbamoyl-ω-methyl-poly(ethylene glycol) (PEG-cholesterol)

[0629]   To a 200-mL round-bottom flask charged with a solution of 2.0 g (0.89 mmol) of 1-[8'-ammino-3',6'-dioxaoctanyl]carbamoyl-ω-methyl-poly(ethylene glycol), 22 mg (0.18 mmol) of 4-dimethylaminopyridine, and 0.93 mL (5.3 mmol) of diisopropylethylamine in 20 mL of anhydrous THF, was added with stirring a solution of 1.20 g (2.67 mmol) of cholesterol chloroformate in 20 mL of anhydrous THF. The resulting reaction mixture was heated to gentle reflux overnight. After cooled, the solvents were removed by rotary evaporation, and the resulting residue was applied onto a silica gel column for purification (methanol/dichloromethane 5:95 to 10:90). The chromatography yielded 2.43 g (91%) of white solid product.

3,4-Ditetradecoxy]benzyl-ω-methyl-poly(ethylene glycol) ether (PEG-DMB)

[0630]   To a 100-mL round-bottom flask charged with a solution of 2.67 g (5.00 mmol) of ditetradecoxylbenzyl alcohol in 20 mL of 1,4-dioxane, was added 20 mL of 4.0 M HCl solution in 1,4-dioxane. The flask was then equipped with a refluxing condenser, which was connected to a sodium bicarbonate solution to absorb any evolved hydrogen chloride gas. After the reaction mixture was heated to 80 for 6 h, thin layer chromatography (dichloromethane as developing solvent) indicated the completion of the reaction. The solvent and the excessive reagent were completely removed under vacuum by rotary evaporation to afford 2.69 g (97%) of gray solid 3,4-ditetradecoxylbenzyl chloride. This crude material was employed directly for the next step reaction without further purification.

[0631]   Poly(ethylene glycol) methyl ether (2.00 g, 1.00 mmol) was dried by co-evaporating with toluene (2×20 mL)

under vacuum. To a solution of the dried poly(ethylene glycol) in 30 mL of anhydrous toluene, was added with stirring 0.17 g (7.2 mmol) of sodium hydride in portions. Gas evolvement took place instantly. The resulting mixture continued to be stirred at 60 for 2 h to ensure the complete formation of oxide. A solution of 0.668 g (1.20 mmol) 3,4-ditetradecoxylbenzyl chloride in 10 mL of anhydrous toluene was then introduced dropwise to the above mixture. The reaction mixture was allowed to stir at 80 overnight. After cooled, the reaction was quenched by the addition of 10 mL of saturated ammonium chloride solution. The resulting mixture was then taken into 300 mL of dichloromethane, washed with saturated ammonium chloride (3×100 mL), dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was purified by flash chromatography (methanol/dichloromethane 2:98 to 5:95) to furnish 1.24 g (49%) of gray solid of the desired product.

Example 17

Preparation of Nanoparticle Encapsulated siNA Formulations

[0632]   siNA nanoparticle solutions were prepared by dissolving siNAs in 25 mM citrate buffer (pH 4.0) at a concentration of 0.9 mg/mL. Lipid solutions were prepared by dissolving a mixture of cationic lipid (e.g., CLinDMA or DOBMA, see structures and ratios for Formulations in Table IV), DSPC, Cholesterol, and PEG-DMG (ratios shown in Table IV) in absolute ethanol at a concentration of about 15 mg/mL. The nitrogen to phosphate ratio was approximate to 3:1.

[0633]   Equal volume of siNA and lipid solutions was delivered with two FPLC pumps at the same flow rates to a mixing T connector. A back pressure valve was used to adjust to the desired particle size. The resulting milky mixture was collected in a sterile glass bottle. This mixture was then diluted slowly with an equal volume of citrate buffer, and filtered through an ion-exchange membrane to remove any free siRNA in the mixture. Ultra filtration against citrate buffer (pH 4.0) was employed to remove ethanol (test stick from ALCO screen), and against PBS (pH 7.4) to exchange buffer. The final liposome was obtained by concentrating to a desired volume and sterile filtered through a 0.2 μm filter.

[0634]   The obtained liposomes were characterized in term of particle size, Zeta potential, alcohol content, total lipid content, nucleic acid encapsulated, and total nucleic acid concentration.

[0635]   All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

[0636]   One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

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[0637] It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims. The present invention teaches one skilled in the art to test various combinations and/or substitutions of chemical modifications described herein toward generating nucleic acid constructs with improved activity for mediating RNAi activity. Such improved activity can comprise improved stability, improved bioavailability, and/or improved activation of cellular responses mediating RNAi. Therefore, the specific embodiments described herein are not limiting and one skilled in the art can readily appreciate that specific combinations of the modifications described herein can be tested without undue experimentation toward identifying siNA molecules with improved RNAi activity.

[0638] The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations that are not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

[0639] In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

TABLE I

Non-limiting examples of Stabilization Chemistries
for chemically modified siNA constructs

Chemistry	pyrimidine	Purine	cap	p = S	Strand
"Stab 00"	Ribo	Ribo	TT at 3'-ends		S/AS
"Stab 1"	Ribo	Ribo	—	5 at 5'-end 1 at 3'-end	S/AS
"Stab 2"	Ribo	Ribo	—	All linkages	Usually AS
"Stab 3"	2'-fluoro	Ribo	—	4 at 5'-end 4 at 3'-end	Usually S
"Stab 4"	2'-fluoro	Ribo	5' and 3'-ends	—	Usually S
"Stab 5"	2'-fluoro	Ribo	—	1 at 3'-end	Usually AS
"Stab 6"	2'-O-Methyl	Ribo	5' and 3'-ends	—	Usually S
"Stab 7"	2'-fluoro	2'-deoxy	5' and 3'-ends	—	Usually S
"Stab 8"	2'-fluoro	2'-O-Methyl	—	1 at 3'-end	S/AS
"Stab 9"	Ribo	Ribo	5' and 3'-ends	—	Usually S
"Stab 10"	Ribo	Ribo	—	1 at 3'-end	Usually AS
"Stab 11"	2'-fluoro	2'-deoxy	—	1 at 3'-end	Usually AS
"Stab 12"	2'-fluoro	LNA	5' and 3'-ends		Usually S
"Stab 13"	2'-fluoro	LNA		1 at 3'-end	Usually AS
"Stab 14"	2'-fluoro	2'-deoxy		2 at 5'-end 1 at 3'-end	Usually AS
"Stab 15"	2'-deoxy	2'-deoxy		2 at 5'-end 1 at 3'-end	Usually AS
"Stab 16"	Ribo	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 17"	2'-O-Methyl	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 18"	2'-fluoro	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 19"	2'-fluoro	2'-O-Methyl	3'-end		S/AS
"Stab 20"	2'-fluoro	2'-deoxy	3'-end		Usually AS
"Stab 21"	2'-fluoro	Ribo	3'-end		Usually AS
"Stab 22"	Ribo	Ribo	3'-end		Usually AS
"Stab 23"	2'-fluoro*	2'-deoxy*	5' and 3'-ends		Usually S
"Stab 24"	2'-fluoro*	2'-O-Methyl*	—	1 at 3'-end	S/AS
"Stab 25"	2'-fluoro*	2'-O-Methyl*	—	1 at 3'-end	S/AS
"Stab 26"	2'-fluoro*	2'-O-Methyl*	—		S/AS
"Stab 27"	2'-fluoro*	2'-O-Methyl*	3'-end		S/AS
"Stab 28"	2'-fluoro*	2'-O-Methyl*	3'-end		S/AS
"Stab 29"	2'-fluoro*	2'-O-Methyl*		1 at 3'-end	S/AS
"Stab 30"	2'-fluoro*	2'-O-Methyl*			S/AS

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TABLE I-continued

Non-limiting examples of Stabilization Chemistries for chemically modified siNA constructs

Chemistry	pyrimidine	Purine	cap	p = S	Strand
"Stab 31"	2'-fluoro*	2'-O-Methyl*	3'-end		S/AS
"Stab 32"	2'-fluoro	2'-O-Methyl			S/AS
"Stab 33"	2'-fluoro	2'-deoxy*	5' and 3'-ends	—	Usually S
"Stab 34"	2'-fluoro	2'-O-Methyl*	5' and 3'-ends		Usually S
"Stab 3F"	2'-OCF3	Ribo	—	4 at 5'-end 4 at 3'-end	Usually S
"Stab 4F"	2'-OCF3	Ribo	5' and 3'-ends	—	Usually S
"Stab 5F"	2'-OCF3	Ribo	—	1 at 3'-end	Usually AS
"Stab 7F"	2'-OCF3	2'-deoxy	5' and 3'-ends	—	Usually S
"Stab 8F"	2'-OCF3	2'-O-Methyl	—	1 at 3'-end	S/AS
"Stab 11F"	2'-OCF3	2'-deoxy	—	1 at 3'-end	Usually AS
"Stab 12F"	2'-OCF3	LNA	5' and 3'-ends		Usually S
"Stab 13F"	2'-OCF3	LNA		1 at 3'-end	Usually AS
"Stab 14F"	2'-OCF3	2'-deoxy		2 at 5'-end 1 at 3'-end	Usually AS
"Stab 15F"	2'-OCF3	2'-deoxy		2 at 5'-end 1 at 3'-end	Usually AS
"Stab 18F"	2'-OCF3	2'-O-Methyl	5' and 3'-ends		Usually S
"Stab 19F"	2'-OCF3	2'-O-Methyl	3'-end		S/AS
"Stab 20F"	2'-OCF3	2'-deoxy	3'-end		Usually AS
"Stab 21F"	2'-OCF3	Ribo	3'-end		Usually AS
"Stab 23F"	2'-OCF3*	2'-deoxy*	5' and 3'-ends		Usually S
"Stab 24F"	2'-OCF3*	2'-O-Methyl*	—	1 at 3'-end	S/AS
"Stab 25F"	2'-OCF3*	2'-O-Methyl*	—	1 at 3'-end	S/AS
"Stab 26F"	2'-OCF3*	2'-O-Methyl*	—		S/AS
"Stab 27F"	2'-OCF3*	2'-O-Methyl*	3'-end		S/AS
"Stab 28F"	2'-OCF3*	2'-O-Methyl*	3'-end		S/AS
"Stab 29F"	2'-OCF3*	2'-O-Methyl*		1 at 3'-end	S/AS
"Stab 30F"	2'-OCF3*	2'-O-Methyl*			S/AS
"Stab 31F"	2'-OCF3*	2'-O-Methyl*	3'-end		S/AS
"Stab 32F"	2'-OCF3	2'-O-Methyl			S/AS
"Stab 33F"	2'-OCF3	2'-deoxy*	5' and 3'-ends	—	Usually S
"Stab 34F"	2'-OCF3	2'-O-Methyl*	5' and 3'-ends		Usually S

CAP = any terminal cap moiety.
All Stab 00–34 chemistries can comprise 3'-terminal thymidine (TT) residues
All Stab 00–34 chemistries typically comprise about 21 nucleotides, but can vary as described herein.
S = sense strand
AS = antisense strand
*Stab 23 has a single ribonucleotide adjacent to 3'-CAP
*Stab 24 and Stab 28 have a single ribonucleotide at 5'-terminus
*Stab 25, Stab 26, and Stab 27 have three ribonucleotides at 5'-terminus
*Stab 29, Stab 30, Stab 31, Stab 33, and Stab 34 any purine at first three nucleotide positions from 5'-terminus are ribonucleotides
p = phosphorothioate linkage

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TABLE II

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time* RNA
A. 2.5 μmol Synthesis Cycle ABI 394 Instrument					
Phosphoramidites	6.5	163 μL	45 sec	2.5 min	7.5 min
S-Ethyl Tetrazole	23.8	238 μL	45 sec	2.5 min	7.5 min
Acetic Anhydride	100	233 μL	5 sec	5 sec	5 sec
N-Methyl Imidazole	186	233 μL	5 sec	5 sec	5 sec
TCA	176	2.3 mL	21 sec	21 sec	21 sec
Iodine	11.2	1.7 mL	45 sec	45 sec	45 sec
Beaucage	12.9	645 μL	100 sec	300 sec	300 sec
Acetonitrile	NA	6.67 mL	NA	NA	NA
B. 0.2 μmol Synthesis Cycle ABI 394 Instrument					
Phosphoramidites	15	31 μL	45 sec	233 sec	465 sec
S-Ethyl Tetrazole	38.7	31 μL	45 sec	233 min	465 sec
Acetic Anhydride	655	124 μL	5 sec	5 sec	5 sec
N-Methyl Imidazole	1245	124 μL	5 sec	5 sec	5 sec
TCA	700	732 μL	10 sec	10 sec	10 sec
Iodine	20.6	244 μL	15 sec	15 sec	15 sec
Beaucage	7.7	232 μL	100 sec	300 sec	300 sec
Acetonitrile	NA	2.64 mL	NA	NA	NA

Reagent	Equivalents: DNA/2'-O-methyl/Ribo	Amount: DNA/2'-O-methyl/Ribo	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time* Ribo
C. 0.2 μmol Synthesis Cycle 96 well Instrument					
Phosphoramidites	22/33/66	40/60/120 μL	60 sec	180 sec	360 sec
S-Ethyl Tetrazole	70/105/210	40/60/120 μL	60 sec	180 min	360 sec
Acetic Anhydride	265/265/265	50/50/50 μL	10 sec	10 sec	10 sec
N-Methyl Imidazole	502/502/502	50/50/50 μL	10 sec	10 sec	10 sec
TCA	238/475/475	250/500/500 μL	15 sec	15 sec	15 sec
Iodine	6.8/6.8/6.8	80/80/80 μL	30 sec	30 sec	30 sec
Beaucage	34/51/51	80/120/120	100 sec	200 sec	200 sec
Acetonitrile	NA	1150/1150/1150 μL	NA	NA	NA

Wait time does not include contact time during delivery.
Tandem synthesis utilizes double coupling of linker molecule.

[0641]

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TABLE III

Structure	NAME	Abbrev.
	Cholesterol	Chol
	Cholest-5-en-3β-tosylate	Chol-OTs
	Cholest-5-en-3β-oxybutan-4-ol	Chol-OBu-OH
	Cholest-5-en-3β-oxypent-3-oxa-an-5-ol	Chol-DEG-OH

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TABLE III-continued

Structure	NAME	Abbrev.
	Cholest-5-ene-3β-oxybutan-4-mesylate	
	Cholest-5-en-3β-oxypent-3 -oxa-an-5-mesylate	
	3-Dimethylamino-1,2-propanediol	
	1-(4,4'-Dimethoxytrityloxy)-3-Dimethylamino-2-propanol	
	3-Dimethylamino-2-(Cholest-5-en-3β-oxybutan-4-oxy)-1-propanol	

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TABLE III-continued

Structure	NAME	Abbrev.
	3-Dimethylamino-2-(Cholest-5-en-3β-oxypent-3-oxa-sn-5-oxy)-1-propanol	
	cis,cis-9,12-octadecadiene-1-ol (linoleyl alcohol)	Lin-OH
	cis,cis-9,12-octadecadiene-1-mesylate (linoleyl mesylate)	Lin-OMs
	3-Dimethylamino-2-(Cholest-5-en-3β-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane	CLinDMA
	3-Dimethylamino-2-(Cholest-5-en-3β-oxypent-3-oxa-sn-5-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane	DEG-CLinDMA

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TABLE IV

Lipid Nanoparticle (LNP) Formulations

Formulation #	Composition	Molar Ratio
L051	CLinDMA/DSPC/Chol/PEG-n-DMG	48/40/10/2
L053	DMOBA/DSPC/Chol/PEG-n-DMG	30/20/48/2
L054	DMOBA/DSPC/Chol/PEG-n-DMG	50/20/28/2
L069	CLinDMA/DSPC/Cholesterol/PEG-Cholesterol	48/40/10/2
L073	pCLinDMA or CLin DMA/DMOBA/DSPC/Chol/PEG-n-DMG	25/25/20/28/2
L077	eCLinDMA/DSPC/Cholesterol/2KPEG-Chol	48/40/10/2
L080	eCLinDMA/DSPC/Cholesterol/2KPEG-DMG	48/40/10/2
L082	pCLinDMA/DSPC/Cholesterol/2KPEG-DMG	48/40/10/2
L083	pCLinDMA/DSPC/Cholesterol/2KPEG-Chol	48/40/10/2
L086	CLinDMA/DSPC/Cholesterol/2KPEG-DMG/Linoleyl alcohol	43/38/10/2/7
L061	DMLBA/Cholesterol/2KPEG-DMG	52/45/3
L060	DMOBA/Cholesterol/2KPEG-DMG N/P ratio of 5	52/45/3
L097	DMLBA/DSPC/Cholesterol/2KPEG-DMG	50/20/28
L098	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 3	52/45/3
L099	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 4	52/45/3
L100	DMOBA/DOBA/3% PEG-DMG, N/P ratio of 3	52/45/3
L101	DMOBA/Cholesterol/2KPEG-Cholesterol	52/45/3
L102	DMOBA/Cholesterol/2KPEG-Cholesterol, N/P ratio of 5	52/45/3
L103	DMLBA/Cholesterol/2KPEG-Cholesterol	52/45/3
L104	CLinDMA/DSPC/Cholesterol/2KPEG-cholesterol/Linoleyl alcohol	43/38/10/2/7
L105	DMOBA/Cholesterol/2KPEG-Chol, N/P ratio of 2	52/45/3
L106	DMOBA/Cholesterol/2KPEG-Chol, N/P ratio of 3	67/30/3
L107	DMOBA/Cholesterol/2KPEG-Chol, N/P ratio of 1.5	52/45/3
L108	DMOBA/Cholesterol/2KPEG-Chol, N/P ratio of 2	67/30/3
L109	DMOBA/DSPC/Cholesterol/2KPEG-Chol, N/P ratio of 2	50/20/28/2
L110	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 1.5	52/45/3
L111	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 1.5	67/30/3
L112	DMLBA/Cholesterol/2KPEG-DMG, N/P ratio of 1.5	52/45/3
L113	DMLBA/Cholesterol/2KPEG-DMG, N/P ratio of 1.5	67/30/3
L114	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 2	52/45/3
L115	DMOBA/Cholesterol/2KPEG-DMG, N/P ratio of 2	67/30/3
L116	DMLBA/Cholesterol/2KPEG-DMG, N/P ratio of 2	52/45/3
L117	DMLBA/Cholesterol/2KPEG-DMG, N/P ratio of 2	52/45/3

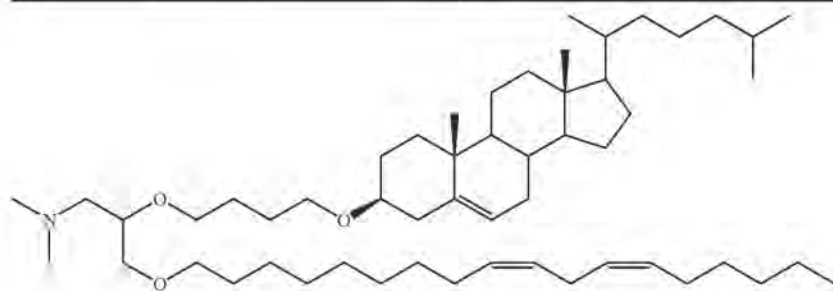N/P ratio = Nitrogen:Phosphorous ratio between cationic lipid and nucleic acid
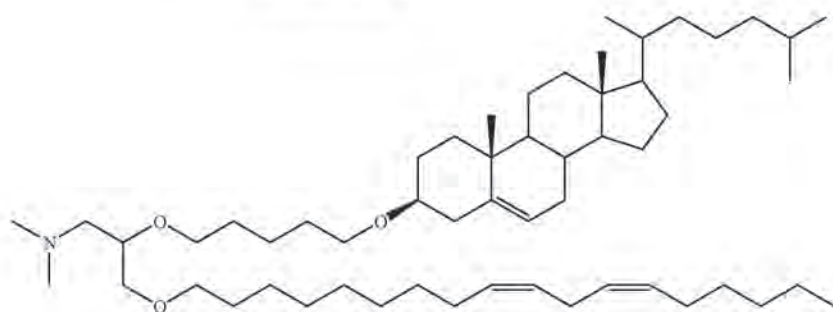
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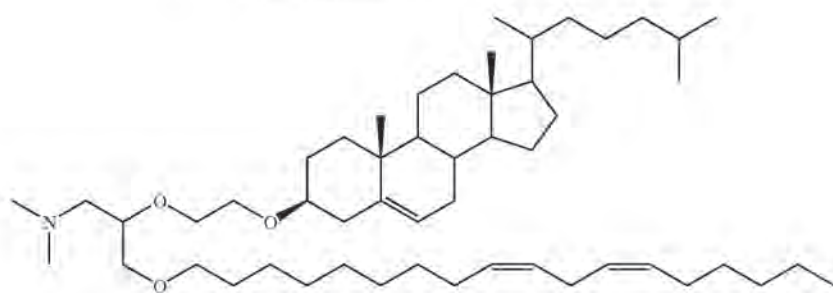US 2006/0240554 A1

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TABLE IV-continued

Lipid Nanoparticle (LNP) Formulations

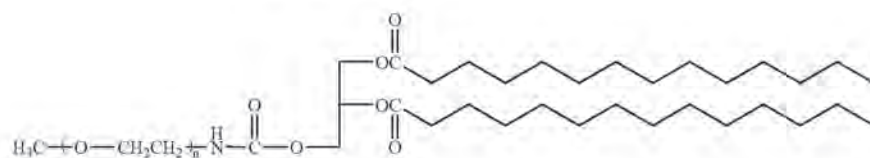| Formulation #	Composition	Molar Ratio

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pCLinDMA structure

eCLinDMA structure

PEG-n-DMG structure
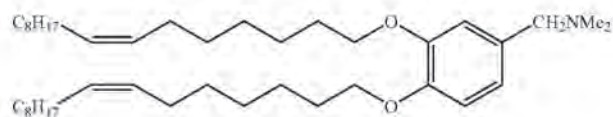
DMOBA structure

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TABLE IV-continued

Lipid Nanoparticle (LNP) Formulations

Formulation #	Composition	Molar Ratio

DMLBA structure

DOBA structure
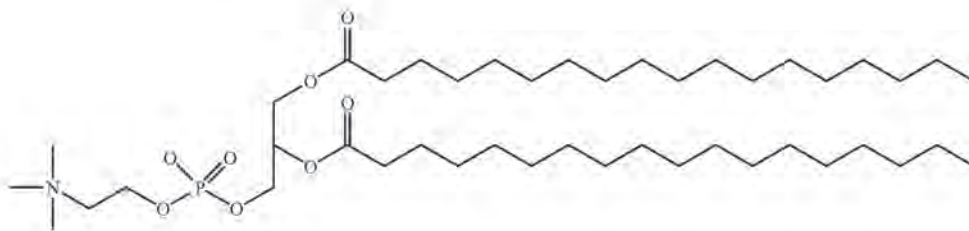
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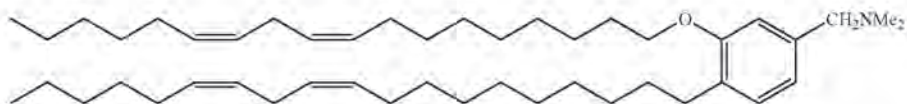

Cholesterol

2KPEG-Cholesterol
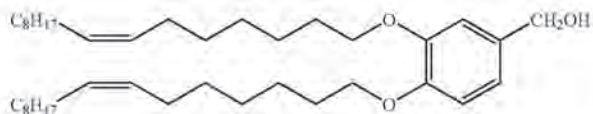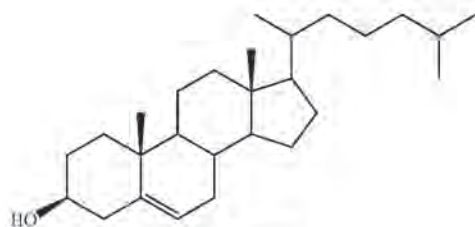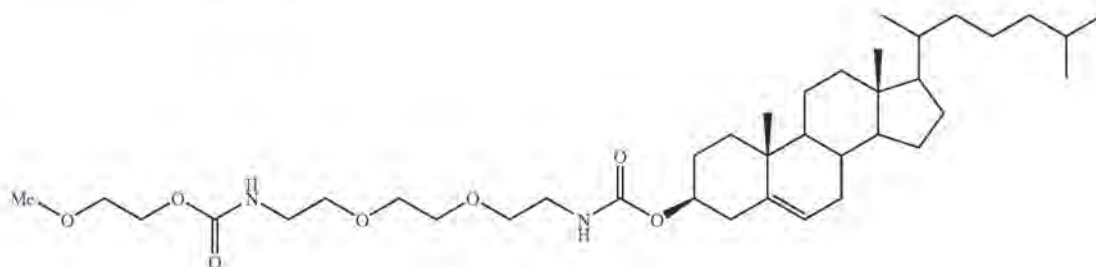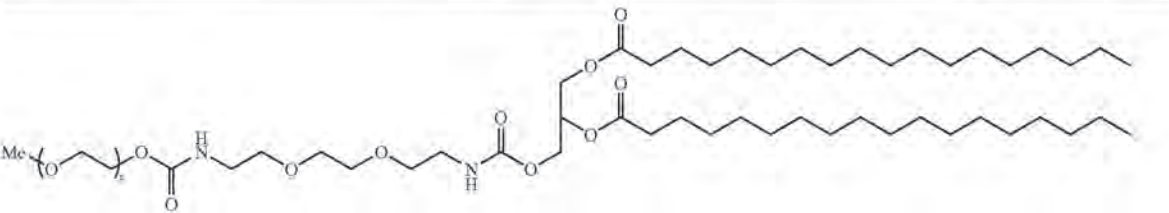
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TABLE IV-continued

Lipid Nanoparticle (LNP) Formulations

Formulation #	Composition	Molar Ratio

2KPEG-DMG

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TABLE V

Cell Line	Tissue	Cell Type	% RNA KD
6.12	spleen	B lymphocyte hybrid	LF2K = 50%
			LNP97 = 90%
			LNP98 = 92%
Raw 264.7	tumor	macrophage/monocyte	LF2K = 85%
			LNP54 = 75%
			LPN98 = 75%

TABLE V-continued

Cell Line	Tissue	Cell Type	% RNA KD
MM14.Lu	normal lung	endothelial/epithelial	LF2K = 90%
			LNP98 = 98%
NIH 3T3	embryo	fibroblast	LF2K = 95%
			LNP51 = 65%
			LPN54 = 65%
			LPN98 = 85%
N/A	lung	primary macrophage	LF2K = 50%
			LNP98 = 65%

[0644]

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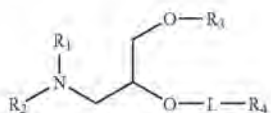
We claim:

1. A compound having Formula CL1:

CLI

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L comprises a C1 to C10 alkyl, alkyl ether, polyether, polyethylene glycol, acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester, or succinyl linker, and R4 comprises cholesterol.

2. A compound having Formula CLII:

CLII

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L comprises a C1 to C10 alkyl, alkyl ether, polyether, polyethylene glycol, acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester, or succinyl linker, and R4 comprises cholesterol.

3. A compound having Formula CLIII:

CLIII

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L comprises a C1 to C10 alkyl, alkyl ether, polyether, poly-

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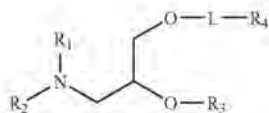
4. A compound having Formula CLIV:

CLIV

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is a C9-C24 aliphatic saturated or unsaturated hydrocarbon, L comprises a C1 to C10 alkyl, alkyl ether, polyether, polyethylene glycol, acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester, or succinyl linker, and R4 comprises cholesterol.
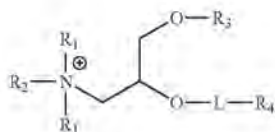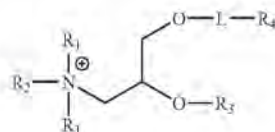
5. A compound having Formula CLV:

CLV

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; and each R3 and R4 is independently linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl.

6. A compound having Formula CLVI:

CLVI

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl; L comprises a C1 to C10 alkyl, alkyl ether, polyether,

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polyethylene glycol, acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester, or succinyl linker, and R4 is cholesterol.

7. A compound having Formula CLVII:

CLVII

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl; L comprises a C1 to C10 alkyl, alkyl ether, polyether, polyethylene glycol, acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester, or succinyl linker, and R4 is cholesterol.
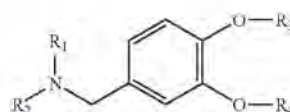
8. A compound having Formula CLVIII:

CLVIII

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; and each R3 and R4 is independently linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl.

9. A compound having Formula CLIX:

CLIX

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl; L comprises a C1 to C10 alkyl, alkyl ether, polyether, polyethylene glycol, acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester, or succinyl linker, and R4 is cholesterol.

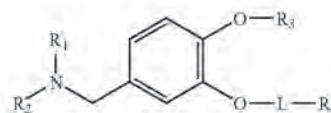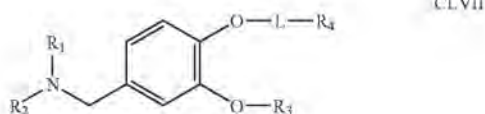10. A compound having Formula CLX:

CLX

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; R3 is linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, or lauroyl; L comprises a C1 t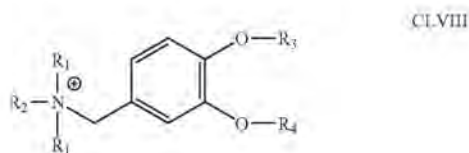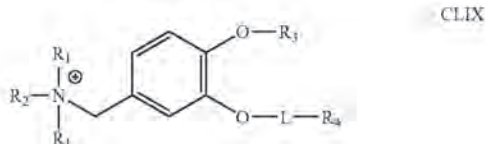o C10 alkyl, alkyl ether, polyether, polyethylene glycol, acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester, or succinyl linker, and R4 is cholesterol.

11. A compound having Formula CLXIX:
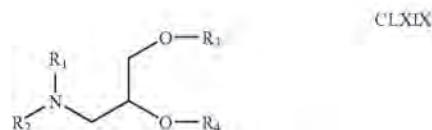
CLXIX

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; and each R3 and R4 is independently linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, lauroyl, or cholesterol.

12. A compound having Formula CLXX:

CLXX

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; and each R3 and R4 is independently linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, lauroyl, or cholesterol.

13. A compound having Formula CLXXIII:

CLXXIII

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wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; each R3 and R4 is independently linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, lauroyl, or cholesterol, and each L is independently a C1 to C10 alkyl, alkyl ether, polyether, polyethylene glycol, acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester, or succinyl linker.
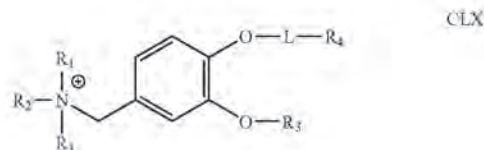
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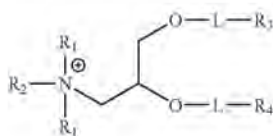
CLXXIV

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; each R3 and R4 is independently linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, lauroyl, or cholesterol, and each L is independently a C1 to C10 alkyl, alkyl ether, polyether, polyethylene glycol, acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester, or succinyl linker.

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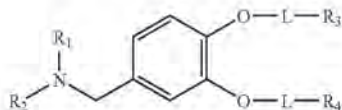
CLXXV

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; each R3 and R4 is independently linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, lauroyl, or cholesterol, and each L is independently a C1 to C10 alkyl, alkyl ether, polyether, polyethylene glycol, acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester, or succinyl linker.

16. A compound having Formula CLXXVI:
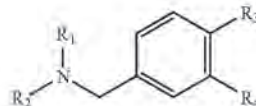
CLXXVI

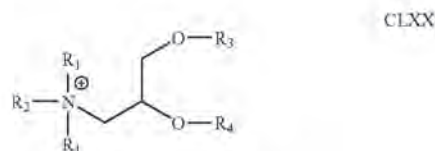wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; each R3 and R4 is independently linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, lauroyl, or cholesterol.

17. A compound having Formula CLXXVII:
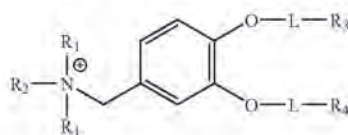
CLXXVII

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; each R3 and R4 is independently linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, lauroyl, or cholesterol, and each L is independently a C1 to C10 alkyl, alkyl ether, polyether, polyethylene glycol, acetal, amide, carbonyl, carbamide, carbamate, carbonate, ester, or succinyl linker.
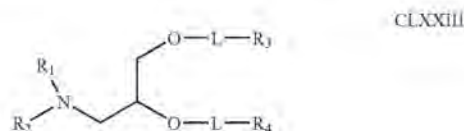
18. A compound having Formula CLXXVIII:
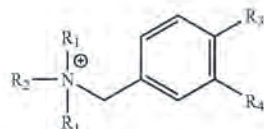
CLXXVIII

wherein each R1 and R2 is independently a C1 to C10 alkyl, alkynyl, or aryl hydrocarbon; each R3 and R4 is independently linoyl, isostearyl, oleyl, elaidyl, petroselinyl, linolenyl, elaeostearyl, arachidyl, myristoyl, palmitoyl, lauroyl, or cholesterol.

**19**. A composition comprising a short interfering nucleic acid (siNA), a cationic lipid having structure as claimed in any of claims **1-18**, a neutral lipid, and a PEG-cholesterol.

20. The composition of claim 19, further comprising cholesterol.

* * * * *

JOINT APPENDIX 42

ACQUIRED DISEASES   RESEARCH ARTICLE

Stabilized plasmid-lipid particles for systemic gene therapy

P Tam[1]*, M Monck[1]*, D Lee[1], O Ludkovski[1], EC Leng[1], K Clow[1], H Stark[2], P Scherrer[3], RW Graham[1] and PR Cullis[1,3]

[1]*Inex Pharmaceuticals Corporation, Burnaby, Canada;* [2]*Institute for Molekularbiologie und Tumorforschung, Marburg, Germany; and* [3]*Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada*

*The structure of 'stabilized plasmid-lipid particles' (SPLP) and their properties as systemic gene therapy vectors has been investigated. We show that SPLP can be visualized employing cryo-electron microscopy to be homogeneous particles of diameter 72 ± 5 nm consisting of a lipid bilayer surrounding a core of plasmid DNA. It is also shown that SPLP exhibit long circulation lifetimes (circulation half-life >6 h) following intravenous (i.v.) injection in a murine tumor model resulting in accumulation of up to 3% of the total injected dose and concomitant reporter gene expression at a distal (hind flank) tumor site. In contrast, i.v. injection of naked plasmid DNA or plasmid DNA–cationic liposome complexes did not result in significant plasmid delivery to the tumor site or gene expression at that site. Furthermore, it is shown that high doses of SPLP corresponding to 175 μg plasmid per mouse are nontoxic as assayed by monitoring serum enzyme levels, whereas i.v. injection of complexes give rise to significant toxicity at dose levels above 20 μg plasmid per mouse. It is concluded that SPLP exhibit properties consistent with potential utility as a nontoxic systemic gene therapy vector. Gene Therapy (2000) **7**, 1867–1874.*

Introduction

Gene therapies for systemic diseases such as cancer or inflammatory disorders clearly require systemic vectors. However, currently available vectors for gene therapy have limited utility for systemic applications. Recombinant virus vectors, for example, are rapidly cleared from the circulation following intravenous injection, limiting potential transfection sites to 'first pass' organs such as the liver.[1,2] Nonviral systems, such as plasmid DNA–cationic liposome complexes, are also rapidly cleared from the circulation, and the highest expression levels are again observed in first pass organs, particularly the lungs.[3–8]

Intravenous administration of chemotherapeutic drugs encapsulated in small (diameter $\leqslant 100$ nm), long-circulating (circulation half-life $t_{\frac{1}{2}} \geqslant 5$ h in murine models) liposomes results in preferential delivery of encapsulated drug to distal tumors due to increased vascular permeability in these regions.[9–11] It therefore follows that intravenous injection of plasmid DNA encapsulated in small, long circulating lipid particles should give rise to preferential delivery of plasmid DNA to tumor sites. Recent work has shown that plasmid DNA can be encapsulated in small (approximately 70 nm diameter) 'stabilized plasmid-lipid particles' (SPLP) that contain one plasmid per particle.[12] These particles contain the 'fusogenic'

lipid dioleoylphosphatidylethanolamine (DOPE), low levels of cationic lipid and are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. Here, we show that the structure of SPLP can be directly visualized employing cryo-electron microscopy to reveal homogeneous particles consisting of plasmid DNA entrapped within a bilayer lipid vesicle. Furthermore, we show that these SPLP exhibit long circulation lifetimes and no evidence of systemic toxicities following i.v. injection in a murine tumor model. Under the experimental conditions employed, approximately 3% of the total injected SPLP dose was delivered to a subcutaneous tumor site and 1.5 % of the total intact plasmid dose could be detected at the tumor site at 24 h. Significant levels of reporter gene expression were observed at the tumor site employing the SPLP system, whereas no expression was observed following i.v. injection of 'naked' plasmid DNA or plasmid DNA–cationic liposome complexes.

Results

SPLP consist of a plasmid trapped inside a bilayer lipid vesicle

Previous work has shown that plasmid DNA can be encapsulated (trapping efficiency approximately 70%) in SPLP by a detergent dialysis procedure employing octylglucopyranoside (OGP).[12] These SPLP are composed of DOPE, 5–10 mol% of the cationic lipid dioleoyldimethylammonium chloride (DODAC) and PEG attached to a ceramide anchor containing an arachidoyl acyl group

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(PEG-CerC$_{20}$). SPLP can be separated from non-encapsulated plasmid by ion exchange chromatography and can then be further purified by density gradient centrifugation to remove empty vesicles produced during the dialysis procedure. On the basis of the size and plasmid-to-lipid ratio of these purified SPLP it was determined that each SPLP contained one plasmid molecule.[12]

Here, we further characterize SPLP structure employing cryo-electron microscopy. Following the procedures summarized in Materials and methods, purified SPLP were prepared from DOPE:DODAC:PEG-CerC$_{20}$ (83:7:10; mol:mol:mol) and pCMVluc, whereas large unilamellar vesicles (LUV) with the same lipid composition were prepared by extrusion of the hydrated lipid mixture through 100 nm pore size filters. As shown in Figure 1a, the cryo-electron micrographs clearly reveal SPLP to con-

sist of a lipid bilayer surrounding an internal structure consistent with entrapped plasmid DNA molecules. Small (diameter approximately 30 nm), empty vesicles formed during the detergent dialysis process[13] that were not removed by density centrifugation do not exhibit such internal structure (see arrows in Figure 1a). This internal structure is also not observed in the LUV produced by extrusion (Figure 1b). It may also be noted that SPLP as detected by cryo-electron microscopy have a remarkably homogeneous size (diameter 72 ± 5 nm), in close agreement with measurements of SPLP diameter employing freeze–fracture electron microscopy (diameter 64 ± 9 nm).[12] The homogeneous size and morphology of SPLP contrasts with the irregular morphology and large size distribution of the extruded vesicles. The narrow size distribution of SPLP was also reflected by quasi-elastic light scattering (QELS) measurements (data not shown) which indicated a mean diameter of 83 ± 4 nm. Plasmid DNA–cationic liposome complexes made from DOPE:DODAC (1:1; mol:mol) LUV exhibited a large, heterogeneous size distribution as determined by QELS (diameter 220 ± 85 nm, data not shown).

SPLP exhibit extended circulation lifetimes, preferential accumulation at tumor sites, and low systemic toxicities following intravenous injection

The next set of experiments was aimed at characterizing the pharmacokinetics and biodistribution of SPLP following i.v. injection into tumor-bearing mice. SPLP were prepared with trace amounts of the lipid label, ^{3}H-cholesteryl hexadecylether (^{3}H-CHE) and were injected at a dose level equivalent to 100 μg plasmid DNA per mouse into C57Bl/6 mice bearing a subcutaneous Lewis lung carcinoma (approximately 200 mg) in the hind flank. The clearance of SPLP from the circulation as assayed by the lipid label (Figure 2a) corresponds to a first order process with a $t_{\frac{1}{2}}$ of 6.4 ± 1.1 h. Relatively low levels of uptake by the lung and liver are observed (Figure 2b and c) whereas approximately 3% of the injected SPLP dose accumulates at the tumor site over 24 h (Figure 2d). Such tumor accumulation levels are comparable with those achieved for small, long-circulating liposomes containing conventional drugs such as doxorubicin, where approximately 5% of the injected dose can be found at 24 h in larger (>0.5 g) tumors.[14] In contrast to the behavior of the SPLP system, ^{3}H-CHE-labeled plasmid DNA–cationic liposome complexes were rapidly cleared from the circulation ($t_{\frac{1}{2}} \ll 15$ min), appearing predominantly in the lung and liver, and less than 0.2% of the injected dose was found at the tumor site at 24 h. The biodistribution of ^{3}H-CHE labeled SPLP and complexes 4 and 24 h following injection are summarized in Table 1. Only trace amounts were detected in kidney, heart and lymph nodes.

The levels of intact plasmid DNA in the circulation and tumor tissue following i.v. injection of naked plasmid DNA, plasmid DNA–cationic lipid complexes and SPLP were analyzed by Southern blot hybridization (Figure 3a, b and c, respectively) and quantified by phosphorimaging analysis (Figure 3d and e). For naked plasmid, less than 0.01% of the injected dose remained intact in the circulation at 15 min, and no intact tumor-associated plasmid could be observed at any time. For plasmid administered in complexes, only a small fraction ($<2\%$) was still intact in the circulation at 15 min and less than 0.2% was found to be intact in tumor tissue at 1 h. In

Figure 1 *Cryo-electron micrographs of (a) purified SPLP and (b) LUV prepared by extrusion. SPLP were prepared from DOPE:DODAC:PEG-CerC$_{20}$ (83:7:10; mol:mol:mol) and pCMVluc and purified employing DEAE column chromatography and density gradient centrifugation. LUV were prepared from DOPE:DODAC:PEG-CerC$_{20}$ (83:7:10; mol:mol:mol) by hydration and extrusion through filters with 100 nm diameter pore size. The arrows in panel (a) indicate the presence of residual 'empty' vesicles formed during the detergent dialysis process that were not removed by the density centrifugation purification step. The bar in panel (b) indicates 100 nm. For details of sample preparation and cryo-electron microscopy see Materials and methods.*

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Figure 2 Pharmacokinetics, tissue distribution and tumor accumulation of SPLP and plasmid DNA–cationic liposome complexes following intravenous administration in tumor-bearing mice as reported by the ^{3}H-CHE lipid marker. The levels of complexes (○) and SPLP (●) in the circulation, the lung, the liver and in Lewis lung tumor tissue are shown in panels (a), (b), (c) and (d), respectively. The accumulations in liver, lung and tumor were corrected for plasma contributions[29] and are expressed as a percentage of the total injected dose.

Table 1 Biodistribution of SPLP and plasmid DNA–cationic liposome complexes in mice 4 and 24 h following i.v. injection

Tissue	% Injected dose (s.e.m.)			
	SPLP		Complexes	
	4 h	24 h	4 h	24 h
Plasma	55.0 (1.7)	6.4 (1.0)	1.7 (0.2)	1.4 (0.3)
Liver	7.0 (0.6)	23.0 (4.3)	35.2 (2.3)	35.1 (3.5)
Lung	0.0 (0.1)	0.2 (0.1)	1.8 (0.8)	0.5 (0.0)
Spleen	0.4 (0.1)	1.6 (0.1)	0.2 (0.2)	0.1 (0.3)
Tumor	0.2 (0.0)	2.8 (0.5)	0.2 (0.1)	0.3 (0.2)

Both SPLP and complexes contained pCMVLuc as well as trace levels of the ^{14}C-labeled CHE lipid marker and were administered at a dose level of 100 μg plasmid per mouse. The biodistribution was measured employing the CHE lipid marker.
s.e.m., standard error of the mean.

contrast, following i.v. injection of SPLP, approximately 85% of the injected plasmid DNA remained in intact form in the circulation at 15 min, and progressively higher levels of intact plasmid accumulated at the tumor site over the time-course of the experiment. The levels achieved at 24 h correspond to approximately 1.5% of the total injected plasmid DNA dose. The circulation half-life of intact plasmid DNA following injection of SPLP was calculated to be 7.2 ± 1.6 h, in good agreement with the circulation half-life of ^{3}H-CHE-labeled SPLP, confirming the highly stable nature of SPLP in the circulation.

Serum enzyme levels of alanine aminotransferase

(ALT) or aspartate aminotransferase (AST) were assayed for evidence of toxicity following i.v. administration of SPLP and plasmid DNA–cationic liposome complexes. Elevated ALT and AST levels are usually associated with liver damage, although elevated AST levels can also indicate systemic tissue damage. Mice receiving SPLP at dose levels as high as 175 μg plasmid DNA per mouse did not have significantly elevated serum levels of ALT and AST (Figure 4a). However, mice receiving doses of plasmid DNA–cationic liposome complexes corresponding to plasmid doses above 20 μg per mouse exhibited progressively higher serum levels of ALT and AST, reaching levels 100-fold above normal levels at plasmid doses of 75 μg (Figure 4b).

Intravenously administered SPLP promote gene expression in a distal tumor

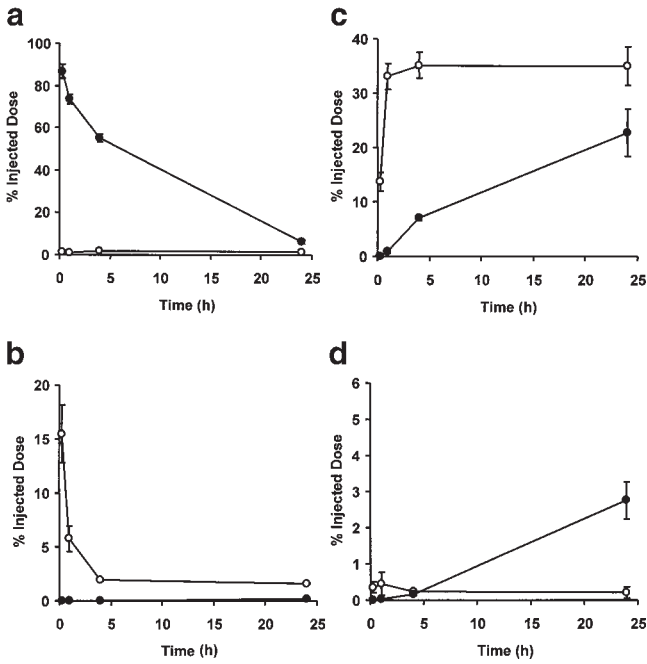It is of obvious interest to determine whether SPLP-mediated delivery of intact plasmid to the tumor site results in transgene expression at that site. Luciferase gene expression in tumor tissue was therefore monitored following i.v. injection of SPLP, naked plasmid DNA and plasmid DNA–cationic liposome complexes at dose levels corresponding to 100 μg plasmid DNA per mouse. This dose level corresponded to the maximum tolerated dose of complexes as evidenced by animal morbidity and mortality. As shown in Figure 5, administration of SPLP results in reporter gene expression at the tumor site, with maximum levels corresponding to 32 pg luciferase per gram of tumor tissue at the 48 h time-point and significant gene expression extending to 96 h after injection. Injection of free plasmid DNA or plasmid DNA–cationic liposome complexes, on the other hand, resulted in no detectable gene expression at the tumor site. It is of interest to note that i.v. administration of complexes did result in transfection in the lung, liver and spleen, whereas administration of SPLP did not result in detectable levels of gene expression in these organs (data not shown). In an attempt to understand why SPLP did not give rise to significant gene expression in the liver, the levels of intact plasmid in the liver 24 h after injection of SPLP into C57BI/6 mice (100 μg plasmid per mouse) bearing a subcutaneous Lewis lung carcinoma were analyzed by Southern blot hybridization. No intact plasmid could be detected in the liver whereas intact plasmid was readily detected at the tumor site (results not shown). This suggests that the ability of SPLP to transfect cells at the tumor site but not in the liver may reflect relatively rapid breakdown of SPLP and associated plasmid following uptake into liver phagocytes (Kuppfer cells), which play a dominant role in clearing liposomal systems from the circulation.[15] Lower gene expression in the liver may also reflect the finding that nonviral vectors such as SPLP transfect dividing cells much more efficiently than non-dividing cells[16] or that Kuppfer cells are less readily transfected than tumor cells.

Discussion

This study demonstrates that SPLP consist of plasmid DNA encapsulated in a bilayer vesicle, and that systemic administration of SPLP results in significant accumulation and transfection at a distal tumor site. There are three important features of these results. The first concerns the structure of SPLP, which represents a major

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Figure 3 Pharmacokinetics and tumor accumulation of plasmid DNA following intravenous administration of naked plasmid, plasmid DNA–cationic liposome complexes and SPLP as reported by a Southern blot analysis. The Southern blot hybridizations shown in panels (a), (b) and (c) result from plasmid DNA isolated from blood and tumor tissue of mice injected with naked plasmid DNA, plasmid DNA–cationic lipid complexes and SPLP, respectively. Each panel shows pCMVluc (2 ng) to indicate the position of intact plasmid DNA. The levels of intact plasmid resulting from i.v. injection of naked plasmid DNA ($\square$), plasmid DNA–cationic liposome complexes ($\bigcirc$) and SPLP ($\bullet$) were quantified for plasma (panel d) and tumor tissue (panel e) by phospor-imaging analysis and converted to mass quantities of plasmid DNA by comparison to a standard curve made from known amounts of plasmid DNA. Tumor accumulations of plasmid were corrected for plasma contributions and expressed as a percentage of the total injected plasmid DNA dose.[29]

advance for plasmid encapsulation in liposomal delivery systems. Second, it is of interest to compare the properties of the SPLP system for systemic gene delivery and distal tumor transfection with the properties of other viral or nonviral gene delivery systems. Finally, the possi-bilities for further optimization of the SPLP system are of interest. We discuss these areas in turn.

The cryo-electron microscopy results presented here establish the structure of SPLP as a plasmid surrounded by a lipid bilayer envelope. This represents the first direct

a

b

Figure 4 Toxicity resulting from i.v. injection into mice of varying amounts of SPLP (panel a) and plasmid DNA–cationic lipid complexes (panel b) as assayed by determining serum levels of the hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (ASP). The serum levels of AST (□) and ALT (■) were measured 24 h after injection.

Figure 5 Transgene expression at a distal tumor site following intravenous injection of naked plasmid DNA (□), plasmid DNA–cationic lipid complexes (○) and SPLP (●). Mice bearing subcutaneous Lewis lung tumors were injected i.v. with doses containing 100 μg of pCMVluc. Tumors were harvested at the indicated times and assayed for luciferase activity. The level of transgene expression reported is normalized for the weight of the tumor tissue.

demonstration that plasmid can be entrapped in small (diameter approximately 70 nm), well defined vesicular systems containing a single plasmid per vesicle. Entrapment of a plasmid such as pCMVluc, which contains 5650 bp, in a supercoiled configuration in a 70 nm diameter vesicle represents a solution for a difficult packing problem. For example, electron micrographs of supercoiled 4.4 kbp plasmids reveals extended lengths of approximately 500 nm and average (two dimensional) diameters in the range of 350 nm, suggesting an average diameter for free supercoiled pCMVluc of approximately 400 nm.[17] The detergent dialysis process clearly involves a partial condensation of entrapped plasmid to allow encapsulation in a 70 nm diameter vesicle. The mechanism of entrapment is not understood in detail, but appears to proceed via association of plasmid with lipid structures formed as intermediates in the detergent dialysis process.[12]

SPLP exhibit extended circulation lifetimes ($t_{\frac{1}{2}}$ approximately 7 h) following i.v. injection, can deliver significant amounts of intact plasmid to a distal tumor site and

enable transgene expression corresponding to 30 pg luciferase per gram tumor at that site with no evidence of toxicity (as indicated 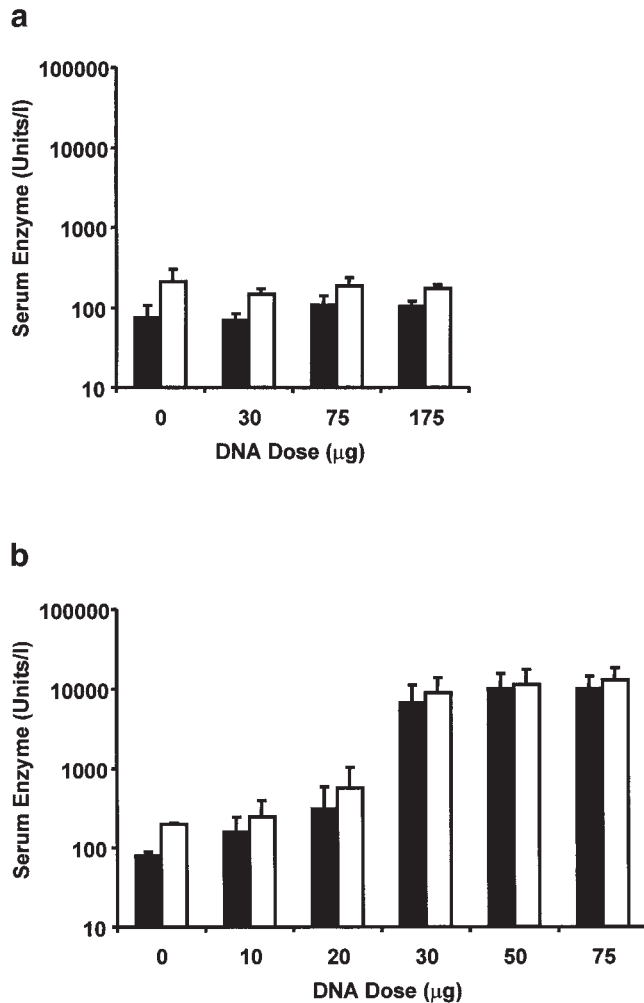by serum enzyme levels). Delivery of approximately 3% of the injected dose of SPLP at a 200 mg tumor site corresponds to more than 1000 plasmid copies per tumor cell, assuming a cell density of 1×10^9 per milliliter. It is of interest to compare these properties with the behavior of other gene delivery systems. In the case of viral vectors, there have been three reports of transgene expression in liver metastases and in a distal tumor following systemic administration of a recombinant vaccinia virus[18,19] and a selectively replicating adenovirus.[20] These viral vectors are replication incompetent in normal nondividing cells but can selectively replicate in tumor cells resulting in transgene expression in tumors and antitumoral efficacy. The major drawback of these viral vectors is the immune response, which occurs within 6 days. In the case of nonviral vectors such as plasmid DNA–cationic polymer 'polyplexes', there is only one report showing transfection of distal tumors following i.v. injection.[21] This work utilized a PEG-containing polyplex that exhibits plasmid circulation half-lives of less than 0.5 h following intravenous injection and gave rise to transfection at a distal tumor site, achieving transfection levels corresponding to approximately 250 pg/g tumor, approximately eight-fold higher than the levels reported here.

With regard to plasmid DNA–cationic liposome complexes ('lipoplexes'), a number of studies have characterized transfection properties following i.v. administration,[3–7] however, only two studies by Xu and co-workers have demonstrated transfection at a distal tumor site.[22,23] In the initial study,[22] less than 5% of the cells at the tumor site were transfected as indicated by immunohistochemical staining, whereas in the second study using transferrin targeted complexes 20–30% of the cells were transfected. The levels of gene expression could not be related to the levels observed here. Issues related to circulation lifetimes, plasmid tumor accumulation and toxicity were not addressed. An additional study[24] has demonstrated the presence of complexes at a distal tumor site following i.v. injection but the levels of

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gene transfer were not measured. In general, i.v. injection of complexes gives rise to high levels of transgene expression in the lungs, with lower levels of expression in the spleen, liver, heart and kidneys. Similar results were observed for the complexes employed in this investigation. The lung expression appears to arise from deposition in lung microvasculature and reflects the rapid clearance of plasmid DNA–cationic lipid complexes from the circulation due to their large size (>200 nm diameter) and high cationic lipid content.[8] This is consistent with the observation that murine B16 tumors seeded in the pulmonary vascular compartment can be transfected by i.v. administered complexes.[3] Finally, as clearly shown in this study, administration of complexes is often associated with significant toxicity.

The final point of discussion concerns the utility of SPLP as a systemic gene therapy vector and the potential for further optimization. As indicated above, despite the delivery of large amounts of intact plasmid to the tumor site, the levels of gene expression observed for the SPLP system are modest, albeit comparable with or superior than can be achieved with other vectors. It is likely that the low levels of transfection reflect low levels of uptake of SPLP into cells at the tumor site due to inhibition of cell association and uptake by the PEG coating.[25] *In vitro* studies have shown that SPLP containing PEG-CerC$_{20}$ are accumulated into cells to a very limited extent, however, the SPLP that are taken up are highly transfection potent.[26] The challenge that faces the next stage of SPLP development is, therefore, to devise methods of enhancing intracellular delivery of SPLP following arrival at the tumor site. There are a number of avenues to explore. First, the dissociation rate of the PEG coating from the SPLP can be modulated by varying the acyl chain length of the ceramide anchor,[12] suggesting the possibility of developing PEG-Cer molecules that remain associated with the SPLP long enough to promote passive targeting to the tumor, but which dissociate quickly enough to allow transfection after arriving at the tumor site. Alternatively, improvements may be expected from inclusion of cell-specific targeting ligands in SPLP to promote cell association and uptake. Finally, the nontoxic properties of SPLP allow the possibility of higher doses. A dose of 100 μg plasmid DNA per mouse corresponds to a dose of approximately 5 mg plasmid DNA per kilogram body weight. This is a relatively low dose level in comparison to small molecules used for cancer therapy, which typically are used at dose levels of 10 to 50 mg per kg body weight.

In summary, we have shown that SPLP consist of plasmid encapsulated in a lipid vesicle. Furthermore, we have demonstrated that, in contrast to naked plasmid or complexes, SPLP exhibit extended circulation lifetimes following intravenous injection, resulting in plasmid accumulation and transgene expression at a distal tumor site in a murine model. The levels of transgene expression achieved are modest, but are comparable or superior to distal tumor expression levels achieved employing other vectors. Further improvements can be expected due to the low toxicity and flexible nature of the SPLP system.

Materials and methods

Lipids and plasmid
1,2-Dioleoyl-3-phosphatidylethanolamine (DOPE) was obtained from Northern Lipids (Vancouver, BC, Canada).

The cationic lipid N,N-dioleyl-N,N-dimethylammonium chloride (DODAC) and 1-O-[2′-(ω-methoxypolyethylene-glycol)succinoyl]-2-N-arachidoylsphingosine (PEG-CerC$_{20}$) were prepared at Inex Pharmaceuticals (Burnaby, BC, Canada) using previously described methods.[27] ^{3}H-labeled cholesteryl hexadecyl ether (CHE) was purchased from Dupont NEN Products (Boston, MA, USA). The pCMVluc plasmid encodes the *Photinus pyralis* luciferase gene under the control of the human CMV immediate–early promoter. Plasmid DNA was propagated in *E. coli* (DH5α) and purified using the alkaline lysis method followed by two rounds of CsCl/ethidium bromide density equilibrium centrifugation.

SPLP and plasmid DNA–cationic liposome complex preparation
Plasmid DNA was encapsulated in SPLP composed of DOPE/DODAC/PEG-CerC$_{20}$ (83:7:10; mol:mol:mol) by the detergent dialysis method.[12] Lipids were dissolved in ethanol and dried to a lipid film in a round-bottom flask. The lipid mixture was resuspended in HBS (5 mM Hepes, 150 mM NaCl, pH 7.5) containing 200 mM OGP and 0.4 mg/ml pCMVluc. The final lipid concentration was 10 mg/ml. When required, ^{3}H-CHE was added to a specific activity of 1.0 μCi/mg total lipid. The mixture of lipid, plasmid and OGP was dialyzed against 4 l of HBS for 2 days with three changes. Untrapped plasmid was removed by DEAE-Sepharose CL-6B chromatography, and plasmid DNA-containing SPLP were purified by sucrose gradient centrifugation (2.5%/5%/10%) in a Beckman SW 28 rotor (16 h at 107 000 *g*) (Beckman, Fullerton, CA, USA). DNA-containing particles banding at the 5%/10% sucrose interface were collected and concentrated by ultrafiltration before the DNA concentration was adjusted to 500 μg/ml. The final lipid composition was determined by HPLC analysis. DNA was quantified by picogreen (Molecular Probes, Eugene, OR, USA) fluorescence of TX-100-solubilized SPLP preparations. Plasmid DNA–cationic liposome complexes were prepared by adding pCMVluc to large unilamellar vesicles (LUV) composed of DOPE:DODAC (1:1; mol:mol) to a final charge ratio (+/−) of 3.0 in 5% glucose. The LUV were prepared by extrusion through 100 nm pore size filters according to standard procedures.[28]

Cryo-electron microscopy
A drop of buffer containing SPLP was applied to a standard electron microscopy grid with a perforated carbon film. Excess liquid was removed by blotting leaving a thin layer of water covering the holes of the carbon film. The grid was rapidly frozen in liquid ethane, resulting in vesicles embedded in a thin film of amorphous ice. Images of the vesicles in ice were obtained under cryogenic conditions at a magnification of 66 000 and a defocus of −1.5 micron using a Gatan cryo-holder in a Philips CM200 FEG electron microscope (Eindhoven, The Netherlands).

Quasi-elastic light scattering
The mean diameter of SPLP was measured by quasi-elastic light scattering (QELS) using a Nicomp Model 370 Sub-Micron particle sizer (Santa Barbara, CA, USA) operated in the particle mode.

Clearance, biodistribution and tumor accumulation of SPLP

Lewis lung carcinoma cells (300 000; ATCC CRL-1642) were implanted subcutaneously in the hind flank of 6-week-old female C57BL/6 mice (Harlan, Indianapolis, IN, USA) and the tumor allowed to grow to approximately 200 mg (12–14 days). Injected materials were then administered intravenously (lateral tail vein injection). All injected doses are reported in micrograms of plasmid DNA per mouse. Blood from animals was collected at the appropriate time-points into blood collection tubes by cardiac puncture. Tumors and organs were quickly removed and frozen at –70°C. Aliquots of plasma separated from blood were analyzed for ^{3}H-CHE by liquid scintillation counting. Plasmid DNA was purified by treating 50 μl plasma with 1× proteinase K buffer (1.0 mg/ml proteinase K, 100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS, pH 8.0). After incubation at 37°C for 3 h, the samples were purified by phenol/chloroform extraction followed by ethanol precipitation. Tumors were homogenized in PBS containing 100 mM EDTA pH 8.0 by using the Fast-Prep 120 homogenizer system (Bio 101, Vista, CA, USA). DNA was purified from an aliquot of the tumor homogenates using the DNAzol reagent according to the manufacturer's guidelines (Life Technologies, Bethesda, MD, USA). The DNA preparations from tumor homogenates were digested with *Eco*RI. DNA samples were subject to electrophoresis through 1.0% agarose gels, transferred to nylon membranes and subjected to Southern blot hybridization using a random primed ^{32}P-labeled restriction fragment from the luciferase gene. Hybridization intensity was quantified using a STORM840 phosphor-imager (Molecular Dynamics, Sunnyvale, CA, USA) and converted to mass of DNA using a standard curve constructed with known amounts of plasmid DNA.

Luciferase assays

Tumor tissue was homogenized in 1× Cell Culture Lysis Reagent (CCLR) (Promega, Madison, WI, USA) using the Fast-Prep 120 homogenizer system (Bio 101). The homogenates were centrifuged at 10 000 g for 2 min before 20 μl of the supernatant was assayed for luciferase activity using the Luciferase Assay System Kit (Promega) on an ML3000 microtiter plate luminometer (Dynex Technologies, Chantilly, VA, USA). Luciferase activities were converted to mass quantities of purified luciferase by comparison with a standard curve generated by assaying known amounts of purified *Photinus pyralis* luciferase enzyme (Boehringer-Mannheim, Laval, PQ, Canada) diluted into untreated tumor extract.

Hepatic release enzyme assays

Plasma from normal C57BL/6 mice injected with SPLP, plasmid DNA–cationic lipid complexes or HBS was recovered 24 h after injection by centrifugation and assayed immediately for ALT or ASP using commercially available kits (Sigma, St Louis, MO, USA).

Acknowledgements

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JOINT APPENDIX 43

GENE DELIVERY

Synthesis and characterization of novel poly(ethylene glycol)-lipid conjugates suitable for use in drug delivery

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Abstract

Liposomal formulations have been used to encapsulate and deliver a wide variety of therapeutic and diagnostic agents. Their circulation can be prolonged by the addition of neutral, hydrophilic polymers such as poly(ethylene glycol) (PEG) to the outer surface. An extended circulation lifetime allows them to take advantage of the enhanced permeability and retention effect (EPR), resulting in increased delivery to target sites. Incorporation of PEG also prevents aggregation and aids in the formation of uniform, small mono-disperse particles. This is often accomplished with the use of PEG-lipid conjugates, PEG molecules with a hydrophobic domain to anchor them into the liposomal bilayer upon formulation.

Here we present data showing that some commonly used PEG-lipids are chemically unstable due to the presence of carboxylic ester bonds. This instability limits their utility in aqueous environments common to many liposomal preparations. To address this problem, we designed and synthesized three alternative PEG-lipids. Using SPLP (PEG-stabilized liposomal vesicles encapsulating plasmid DNA) as a model system, we investigated the properties of the novel PEG-lipids. An accelerated stability study was conducted at 37 °C for 42 days to confirm chemical stability and an in vivo model was used to assess the pharmacokinetics, toxicity and activity of the SPLP. We show that the novel PEG-lipids are more stable in liposomal formulation, less toxic upon systemic administration, and accordingly, are suitable replacements for the PEG-lipids described previously.

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Keywords: Liposome; SPLP; SNALP; PEG; Stability

1. Introduction

Through liposomal encapsulation, it is possible to obtain particles with the small size, low surface charge and increased serum stability necessary to increase circulation lifetime and take advantage of the "enhanced permeation and retention" (EPR) effect [1–3]. This effect occurs where fenestrated vasculature permits the extravasation and increased accumulation of encapsulated material at target sites such as tumors, sites of infection or inflammation [4–6].

Nucleic acid based drugs acquire other benefits from liposomal encapsulation [7–11]. When administered systemically in unprotected form, nucleic acids suffer from poor pharmacokinetics due to rapid degradation by intravascular nucleases. Early liposomal strategies utilized lipoplex, electrostatic complexes formed between cationic lipids and negatively charged nucleic acids [12]. However, unshielded cationic lipoplexes themselves possess poor physico-chemical characteristics for systemic delivery. Their positive surface charge leads to non-specific interaction with anionic species in the blood, resulting in rapid clearance by the reticulo-endothelial system (RES) [13–15].

This problem can be overcome through the use of hydrophilic polymers attached to the particle's surface. Most commonly PEG has been used [16,17], although other polymers have been described [18,19]. However, it has been shown that the presence of PEG can affect the intracellular delivery and trafficking of non-viral vectors, resulting in lower gene expression [20]. To this end, many groups have devised strategies to ensure that the presence of PEG is transient. One strategy involves the use of exchangeable PEG-lipids that rely on slow diffusion from the particle surface at a rate determined by the size of their lipid anchors [9,21]. Examples include PEG-

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phosphatidyl ethanolamines (PEG-PE) [22], PEG-Ceramides (PER-Cer) [21], SAINT-PEGs [23] and PEG-succinoyl diacylglycerols (PEG-S-DAGs) [9]. Another strategy uses PEG-disulphide lipids that possess a disulphide bond between the polymer and the lipid anchor that is cleaved by thiolytic agents in the reductive environment of the endosome [24,25]. Similarly, vinyl ether PEG-lipids [26] and orthoester PEG-lipids [27–29] have been developed using chemical linkages that are sensitive to the reduced pH of the endosomal compartment.

Recently published data shows that when administering repeated doses of liposomally encapsulated, immunostimulatory nucleic acids, a strong, long-lived antibody response can be generated against PEG, a result of the powerful adjuvant effect of the nucleic acid payload [30,31]. While this effect has in the past been attributed to the immunostimulatory CpG motifs of bacterial pDNA [32], CpG free phosphorothioate ODN [33] and siRNA [34] have also been shown to be immunostimulatory. This impacts the potential for repeat administration of PEGylated nucleic acid delivery systems, causing a loss of disease site targeting, accelerated blood clearance and acute hypersensitivity upon subsequent administration. It has been shown that the use of PEG-lipids with a smaller C_{14} lipid anchor, thereby increasing the rate of dissociation of the PEG from the particle, abrogates this deleterious effect [30,31]. In this regard, there may be advantages to a strategy using exchangeable PEG layers, rather than those that are permanently bound or not cleaved until entry to the endosome.

Our characterization of the immune response to PEG-lipids [31] coincided with our observation that PEG-succinoyl distearylglycerol (PEG-S-DSG)-containing SPLP in a controlled, long-term stability study exhibited a steadily decreasing concentration of PEG-lipid over time (Fig. 1), ultimately leading to particle destabilization and aggregation. This led us to believe that PEG-S-DSG was chemically unstable, an undesirable attribute for a component of a potential pharmaceutical product. An examination of commercially available alternatives and the literature yielded no acceptable substitutes for PEG-S-DSG. Therefore, we set out to design, synthesize and characterize novel replacements with the aim of preparing SPLP that are at least as efficacious as those containing the original PEG-S-DSG.

2. Materials and methods

2.1. Materials and analyses

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). 1,2-dioleyloxy-*N,N*-dimethyl-3-aminopropane (DODMA) [35], PEG-S-DAGs [9] and PEG-CerC$_{20}$ [21] were prepared as previously described. ^{3}H-labelled CHE was obtained from Perkin-Elmer (Boston, MA, USA). The Picogreen Quantitation Assay and Kit was obtained from Molecular Probes (Eugene, OR, USA). GPR grade solvents were purchased from VWR Scientific (Edmonton, AB, Canada). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada). The pCMVluc plasmid, encoding the luciferase reporter gene under the control of the cytomegalovirus promoter, was manufactured as described previously [36]. [1]H nuclear magnetic resonance (NMR) spectrometry was performed by Spectral Data Services, Inc. (IL, USA). Elemental analysis (CHN) was performed by Canadian Microanalytical Service Ltd. (BC, Canada).

2.2. SPLP preparation

SPLP at a total lipid concentration of approximately 10 mg/mL were prepared using the method of spontaneous vesicle formation by ethanol dilution, as described previously [37]. The lipid composition was DSPC : cholesterol : PEG-lipid : DODMA (20:55:10:15 molar ratio). Nucleic acid encapsulation was determined using a PicoGreen assay, and encapsulation efficiency calculated by comparing fluorescence in the presence and absence of Triton X-100 [37]. Picogreen fluorescence was measured using a Varian Eclipse Spectrofluorometer (Varian Inc., CA, USA). Particle size was determined using a Malvern Instruments Zetasizer 3000HSA (Malvern, UK). SPLP (40 μL) were diluted with 4 mL of phosphate buffered saline (PBS (150 mM NaCl, 10 mM Phosphate, pH 7.4 buffer)). Intensity-weighted, Gaussian distribution analysis was used to determine mean vesicle diameters and population standard deviations.

Fig. 1. (A) Accelerated stability study of SPLP lipids at 40 °C. Lipid concentrations were determined by HPLC analysis using an evaporative light scattering detector. PEG-S-DSG, possessing four carboxylic ester bonds, exhibited a pronounced tendency to degrade. DSPC was also unstable at 40 °C. (B) Stability study of PEG-S-DSG in SPLP at 40, 25 and 5 °C. Data-points are the mean of 2 analyses. Error shown is the percent relative standard deviation of six analyses of a standard containing all four lipids.

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Polydispersity was reported using 'Contin' mode of the instruments PCS software.

2.3. SPLP stability studies and HPLC analysis

SPLP formulations were incubated at relevant temperatures (5, 25, 37 or 40 °C) for the duration of the study in question (up to 12 months for the long-term study, 42 days for the accelerated study). Samples (100 µL) were withdrawn using a Gilson pipette and prepared for HPLC analysis by diluting 1:19 in ethanol. This dissolved the SPLP lipid bilayer and resulted in precipitation of the nucleic acids and buffer salts. Precipitates were removed by filtering through a 13 mm Acrodisc 0.45 µm syringe filter (Pall Corp., Ann Arbor, MI). A Waters Alliance 2695 HPLC (Waters Ltd., ON, Canada) with an ACE C_8, 4.6×250 mm, 5 µm column (Canadian Life Science, ON, Canada), was used for analysis with sample injection volumes of 20 µL. Sample and column temperatures were 20 and 40 °C, respectively. The mobile phase gradient varied linearly from 80:20 A:B to 83:17 A:C (where A = methanol, B = 10 mM ammonium bicarbonate, pH 8.0, C = tetrahydrofuran (THF)) over a time period of 18 min. The mobile phase flow rate was 1.1 mL per min. A 6-min hold at the final conditions was followed by a 21-min re-equilibration period for the column (equating to a total run time of 45 min). An Alltech Evaporative Light Scattering Detector 2000 (Alltech Associates Inc., IL, USA) was used for detection, with the drift tube temperature set at 70 °C and gas flow rate set at 1.8 L per min. A standard curve was generated for each lipid. System suitability was determined prior to each run by analysis of a standard sample containing all four lipids. When the relative standard deviation (RSD) of six repeat analyses was less than 2.0% for each lipid, the run was considered acceptable. For the long-term stability study, $n=2$. For the accelerated study, $n=3$.

2.4. In vivo transfection studies

Neuro-2a neuroblastoma cells were cultured in Minimum Essential Medium (MEM; Invitrogen, ON, Canada), supplemented with 10% fetal bovine serum (FBS; Invitrogen, ON, Canada) at 37 °C with 5% CO_2. 5-week-old male A/J mice (Harlan, IN, USA) were inoculated subcutaneously in the hind flank with 1.5×10^6 Neuro-2a cells 13 days prior to SPLP treatment. SPLP containing 50 µg DNA in a 100 µL injection volume or 100 µL PBS were administered by lateral tail vein injection. Mice were sacrificed 48 h after treatment. Tumor, liver, lungs, spleen and heart tissues were subject to analysis for luciferase gene expression as described previously [9]. For the experiment in Fig. 5, $n=6$. For the experiment in Fig. 7B, $n=4$.

2.5. Toxicity studies

5-week-old male A/J mice (Harlan, IN, USA) were treated with SPLP containing 50 µg DNA in a 100 µL injection volume or 100 µL PBS, administered by lateral tail vein injection. Animals were sacrificed 48 h after treatment. Serum was assayed for alanine aminotransferase (ALT) and aspartate amiotransferase

(AST) by the Central Laboratory for Veterinarians (Langley, BC, Canada). For this experiment, $n=3$.

2.6. Pharmacokinetic studies

SPLP containing tritiated cholesteryl hexadecyl ether (^{3}H-CHE) (1.0 µCi/mg of total lipid) in the lipid solution were prepared as in Section 2.2. Injections containing 50 µg DNA in a 100 µL volume were administered to 5-week-old male A/J mice (Harlan, IN, USA) by lateral tail vein injection. At appropriate time points, ^{3}H-CHE in whole blood was determined by liquid scintillation counting, using Picofluor 15 and a Beckman LS6500 (Beckman Instruments, CA, USA). For this experiment, $n=4$.

2.7. Synthesis of lipid anchors

The synthesis of the C_{18} lipid anchor is described below. The C_{14} anchor was made in an analogous fashion, substituting an equimolar amount of 1-bromotetradecane for the 1-bromooctadecane in the reaction to make the analogous compound **1**.

2.7.1. Preparation of 1,2-distearyloxy-3-allyloxypropane (1)

Benzene (250 mL) was added to 95% sodium hydride (11.4 g, 450.0 mmol), and the flask was sealed and flushed with nitrogen. A solution of 3-allyloxy-1,2-propanediol (6.6 g, 50.0 mmol) in benzene (75 mL) was added to the flask. 96% 1-bromooctadecane (41.7 g, 120.0 mmol) was added and the reaction left to reflux overnight under nitrogen. The mixture was cooled to room temperature and excess sodium hydride slowly quenched with ethanol. The solution was transferred to a separatory funnel with benzene (250 mL) and washed with distilled water (2×200 mL) and brine (1×200 mL). The organic fractions were combined, dried over magnesium sulfate and concentrated. The crude product was purified by flash column chromatography (1–5% ether in hexane) to yield compound **1** as a colourless wax (21.0 g, 66%, R_f=0.35 (5% ether in hexane)). ^{1}H NMR (400 MHz), δ_H: 5.90 (m, 1H, CH_2=CH), 5.21 (m, 2H, CH_2=CH), 4.01 (m, 2H, CH_2=CHCH_2), 3.61–3.40 (m, 9H, $CH_2CH(OCH_2)CH_2OCH_2$), 1.61–1.51 (m, 4H, OCH_2CH_2), 1.38–1.20 (m, 60H, $CH_{2(stearyl)}$), 0.88 (t, 6H, CH_3, J=6.8 Hz).CHN found; C 79.06, H 13.15 ($C_{42}H_{84}O_3$=C 79.18; H 13.29; O 7.53).

2.7.2. Preparation of 1,2-distearyloxypropan-3-ol (2)

Compound **1** (21.0 g, 33 mmol) was dissolved in ethanol (250 mL) and trifluoroacetic acid (20 mL) and tetrakis (triphenylphosphine) palladium(0) (5.0 g, 4.3 mmol) added. The reaction mixture was refluxed under nitrogen overnight. The solvent was removed by rotary evaporator and the crude product purified by flash column chromatography (100% dichloromethane (DCM)), to yield compound **2** as a colourless wax (18.7 g, 95%, R_f=0.4 (chloroform)). ^{1}H NMR (400 MHz), δ_H: 3.75–3.67 (m, 1H, OCH), 3.67–3.47 (m, 8H, OCH_2), 2.26 (s, 1H, OH), 1.62–1.50 (m, 4H, OCH$_2CH_2$), 1.38–1.20 (m, 60H, $CH_{2(stearyl)}$), 0.88 (t, 6H, CH_3, J=6.8 Hz). CHN found; C 79.06, H 13.25 ($C_{39}H_{80}O_3$=C, 78.46; H, 13.51; O, 8.04).

2.7.3. Preparation of N-(2,3-distearyloxypropyl)phthalimide (3)

97% methanesulphonic anhydride (11.3 g, 62.8 mmol) was dissolved in DCM (anhydrous, 100 mL) and pyridine (anhydrous, 5.0 g, 62.8 mmol) slowly added. A solution of compound **2** (18.7 g, 31.4 mmol) in DCM (anhydrous, 100 mL) was added and the reaction stirred overnight at room temperature. The reaction mixture was washed with distilled water (2×200 mL) and brine (1×200 mL), dried over magnesium sulphate, and concentrated to yield the mesylate as a colourless wax ($R_f = 0.8$ (chloroform)). The mesylate and potassium phthalimide (15.7 g, 85.0 mmol) were added to N,N-dimethylformamide (DMF) (anhydrous, 500 mL) and the mixture stirred at 70 °C overnight under nitrogen. DMF was removed by rotary evaporator attached to a high vacuum pump and the residue triturated in chloroform (300 mL). The suspension was filtered and the filtrate washed (distilled water (2×200 mL) and brine (1×200 mL), dried (magnesium sulphate) and concentrated. The crude product was purified by flash column chromatography (100% DCM) to yield compound **3** as a colourless wax (19.5 g, 86%, $R_f = 0.65$ (DCM)). ^{1}H NMR (400 MHz), δ_H: 7.85 (dd, 2H, $CH_{(arom\ 3,\ 6)}$, $J_{ortho} = 5.5$ Hz, $J_{meta} = 3.0$ Hz), 7.71 (dd, 2H, $CH_{(arom\ 4,\ 5)}$), 3.90–3.70 (m, ^{3}H, NCH_2CH), 3.63–3.38 (m, 6H, OCH_2), 1.54–1.37 (m, 4H, OCH_2CH_2), 1.38–1.06 (m, 60H, $CH_{2(stearyl)}$), 0.88 (t, 6H, CH_3, $J = 6.9$ Hz). CHN found; C 77.76, H 11.69, N 1.86 ($C_{47}H_{83}NO_4 =$ C 77.74, H 11.52, N 1.93, O 8.81).

2.7.4. Preparation of 1,2-distearyloxypropyl-3-amine (4)

Phthalimide deprotection was performed as described previously [38]. Briefly, compound **3** (19.5 g, 26.9 mmol) was dissolved in ethanol (500 mL), hydrazine monohydrate (40 mL, 825 mmol) added and the reaction refluxed overnight. A pale yellow precipitate developed so the suspension was filtered and the filtrate evaporated under reduced pressure. The crude product was purified by flash column chromatography (0–5% MeOH–CHCl$_3$) to give compound **4** as a pale yellow wax (13.1 g, 82%, $R_f = 0.45$ (8% methanol (MeOH) in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 3.65–3.58 (m, 1H, NCH_2CH), 3.52–3.32 (m, 6H, OCH_2), 2.79 (ABX, 2H, $NCH_{2\ (A\ and\ B)}$, $J_{AB} = 13.2$ Hz, $J_{AX} = 3.7$ Hz, $J_{BX} = 6.4$ Hz), 1.62–1.51 (m, 4H, OCH_2CH_2), 1.40–1.20 (m, 62H, NH_2, $CH_{2(stearyl)}$), 0.88 (t, 6H, CH_3, $J = 6.8$ Hz). CHN found; C 78.69, H 13.42, N 2.66 ($C_{39}H_{81}NO_2 =$ C, 78.58; H, 13.70; N, 2.35; O, 5.37).

2.8. Synthesis of PEG-lipids

2.8.1. Preparation of methoxy PEG$_{2000}$ acetic acid (5)

Methoxy poly(ethylene glycol)$_{2000}$ (Me-PEG$_{2000}$-OH) (20.0 g, 10 mmol) was added to a solution of sodium dichromate (3.0 g, 10 mmol) in 10% sulfuric acid (200 mL) and stirred at room temperature overnight. The product was extracted with chloroform (3×250 mL) and the organic fraction combined, washed with 1 M sodium hydroxide (250 mL) and evaporated to yield a pale blue wax. This crude material was purified by flash column chromatography (0–15% MeOH–CHCl$_3$) to give compound **5** as a colourless solid (8.0 g, 38.7%, $R_f = 0.3$ (13% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 4.0–3.9 (s, 2H, CH_2CO_2H), 3.7–3.6 (m, ~ 180H, $OCH_{2(PEG)}$), 3.38 (s, ^{3}H, CH_3O).

2.8.2. Preparation of N-(1,2-distearyloxypropyl) methoxy poly (ethylene glycol)$_{2000}$ acetamide (PEG-A-DSA) (6)

Compound **5** (6.8 g, 3.4 mmol) was dissolved in benzene (80 mL) and oxalyl chloride (3.4 mL, 20 mmol) slowly added. The solution was stirred for 2 h prior to solvent removal by rotary evaporator. Compound **4** (2.15 g, 3.6 mmol), DCM (anhydrous, 80 mL) and triethylamine (TEA) (3 mL, 20 mmol) were added and the reaction was stirred for 48 h. The solution was acidified via the addition of a 1% solution of hydrochloric acid (HCl) (250 mL) with agitation. The organic layer was collected, dried over magnesium sulphate and concentrated to yield a pale yellow solid. The crude product was purified by flash column chromatography (0–7% MeOH in CHCl$_3$), then lyophilized to yield PEG-A-DSA as a colourless solid (3.4 g, 38%, $R_f = 0.5$ (10% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 7.10–7.04 (m, 1H, NH), 4.00 (s, 2H, $CH_2C(O)NH$), 3.7–3.6 (m, ~ 180H, $OCH_{2(PEG)}$), 3.60–3.39 (m, 9H, $NCH_2CH(OCH_2)CH_2OCH_2$), 3.38 (s, ^{3}H, CH_3O), 1.60–1.50 (m, 4H, $OCH_2CH_{2(stearyl)}$), 1.38–1.20 (m, 60H, $CH_{2(stearyl)}$), 0.88 (t, 6H, CH_2CH_3, $J = 6.8$ Hz).

2.8.3. Preparation of methoxy poly(ethylene glycol)$_{2000}$ mesylate (ME-PEG$_{2000}$-OMs) (7)

Pyridine (3.8 mL, 47.0 mmol) was added slowly to a solution of 97% methanesulphonic anhydride (8.2 g, 47.1 mmol) in DCM (anhydrous, 80 mL). A solution of Me-PEG$_{2000}$-OH (31.5 g, 15.5 mmol) in DCM (anhydrous, 120 mL) was added and the reaction stirred overnight. The reaction mixture was washed with distilled water (2×200 mL) and brine (1×200 mL), dried over magnesium sulphate, and concentrated. Purification by flash column chromatography (0–10% MeOH–CHCl$_3$) yielded compound **7** as a colourless solid (30.1 g, 92.8%, $R_f = 0.4$ (5% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 4.38 (t, ^{3}H, CH_2OS, $J = 4.5$ Hz), 3.79–3.74 (m, 2H, CH_2CH_2OS), 3.7–3.6 (m, ~ 180H, $OCH_{2(PEG)}$), 3.38 (s, ^{3}H, CH_3O), 3.08 (s, ^{3}H, $OS(O)_2CH_3$).

2.8.4. Preparation of 1-amino methoxy poly(ethylene glycol) (Me-PEG$_{2000}$-NH$_2$) (8)

Me-PEG$_{2000}$-NH$_2$ was prepared as previously described [39]. Briefly, compound **7** (10 g, 5 mmol) was dissolved in a concentrated solution of aqueous ammonia (400 mL), sealed and left to stir for 72 h. The product was extracted with DCM (3×300 mL) and the combined organic fractions dried over MgSO$_4$ and concentrated. The product was crystallized from diethyl ether to yield compound **9** as a colourless solid (9.3 g, 93%, $R_f = 0.15$ (10% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 3.7–3.6 (m, ~ 180H, $OCH_{2(PEG)}$), 3.58–3.53 (m, 2H, $CH_2CH_2NH_2$), 3.38 (s, ^{3}H, CH_3O), 2.90 (t, 2H, CH_2NH_2, $J = 5.2$ Hz), 2.2–1.9 (bs, 2H, NH_2).

2.8.5. Preparation of methoxy poly(ethylene glycol) succinimide (Me-PEG$_{2000}$-Sn) (9)

Succinic anhydride (3.8 g, 38.1 mmol) was added to a solution of compound **8** (9.0 g, 4.4 mmol) in pyridine (anhydrous, 100 mL)

and the reaction stirred overnight. The pyridine solvent was removed under reduced pressure, and the residue dissolved in distilled water (100 mL) and acidified with HCl. Crude product was extracted with DCM (3×100 mL), dried over magnesium sulphate and concentrated. Purification by flash column chromatography (0–10% MeOH–CHCl$_3$) yielded compound **9** as a colourless solid (5.7 g, 61%, R_f=0.3 (10% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 6.69 (m, 1H, N*H*), 3.7–3.6 (m, ~180H, OC*H*$_{2(PEG)}$), 3.58–3.53 (m, 2H, C*H*$_2$CH$_2$NH), 3.45 (quint, 2H, CH$_2$C*H*$_2$NH, *J*=5.0 Hz), 3.38 (s, ^{3}H, CH$_3$O), 2.70–2.61 (m, 2H, C*H*$_2$CO$_2$H), 2.57–2.50 (m, 2H, C*H*$_2$CH$_2$CO$_2$H).

2.8.6. Preparation of N-[(methoxy poly(ethylene glycol)$_{2000}$ succinimidyl]-1,2-distearyloxlpropyl-3-amine (PEG-S-DSA) (10)

N-hydroxysuccinamide (360 mg, 3 mmol) and compound **9** (3.1 g, 1.5 mmol) were dissolved in chloroform (anhydrous, 30 mL). A solution of 1,3-dicyclohexyl-carbodiimide (DCC) (490 mg, 2.25 mmol) in chloroform (anhydrous, 10 mL) was added, and the reaction stirred for 1 h. A separate solution of compound **4** (900 mg, 1.5 mmol) and TEA (0.9 mL, 6 mmol) in chloroform (anhydrous, 10 mL) was added and the reaction for a further hour. The solution was filtered through a bed of Celite and distilled water added (50 mL). The mixture was acidified with concentrated HCl, then washed again with distilled water (2×50 mL) and brine (50 mL). Organic fractions were combined, dried over magnesium sulphate and concentrated. The product was purified by flash column chromatography (0–7% MeOH–CHCl$_3$) to yield PEG-S-DSA, compound **10**, as a colourless solid (3.5 g, 88%, R_f=0.65 (12% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 6.84–6.79 (m, 1H, N*H*CH$_2$CH), 6.40–6.34 (m, 1H, CH$_2$CH$_2$N*H*), 3.7–3.6 (m, ~180H, OC*H*$_{2(PEG)}$), 3.60–3.39 (m, 13H, OC*H*$_2$CH$_2$CN, NC*H*$_2$CH(OC*H*$_2$)CH$_2$OC*H*$_2$), 3.38 (s, 3H, CH$_3$O), 2.54–2.49 (m, 2H, C*H*$_2$CH$_2$CO$_2$H), 1.60–1.51 (m, 4H, OCH$_2$C*H*$_{2(stearyl)}$), 1.40–1.20 (m, 60H, CH$_{2(stearyl)}$), 0.88 (t, 6H, C*H*$_3$, *J*=6.9 Hz).

2.8.7. N-[(methoxy poly(ethylene glycol)$_{2000}$)carbamyl]-1,2-distearyloxlpropyl-3-amine (PEG-C-DSA) (11)

Diphosgene (2.0 mL, 16.7 mmol) was added to a solution of PEG$_{2000}$ methyl ether (10.0 g, 5 mmol) in DCM (anhydrous, 100 mL) and stirred under nitrogen at room temperature for 3 h. DCM and excess diphosgene were then removed under reduced pressure and compound **4** (4.2 g, 7 mmol) added. The flask was flushed with nitrogen, then DCM (anhydrous, 150 mL) and TEA (1.4 mL) added prior to stirring overnight. The solution was diluted with DCM (100 mL), washed (1% HCl (1×200 mL), water (1×200 mL) and brine (1×200 mL)), dried over magnesium sulphate and concentrated. Purification by flash column chromatography (1.5–6.0% MeOH in CHCl$_3$) followed by lyophilization afforded PEG-C-DSA, compound **11**, as a colourless solid (11.9 g, 90%, R_f=0.35 (8% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 5.16–5.08 (m, 1H, N*H*), 4.25–4.19 (m, 2H, C*H*$_2$OC(O)), 3.7–3.6 (m, ~180H, OC*H*$_{2(PEG)}$), 3.60–3.39 (m, 9H, NC*H*$_2$CH(OC*H*$_2$)CH$_2$OC*H*$_2$), 3.38 (s, 3H, CH$_3$O), 1.60–1.50 (m, 4H, OCH$_2$C*H*$_{2(stearyl)}$), 1.38–1.20 (m, 60H, CH$_{2(stearyl)}$), 0.88 (t, 6H, CH$_2$C*H*$_3$, *J*=6.8 Hz).

2.8.8. N-[(methoxy poly(ethylene glycol)$_{2000}$)carbamyl]-1,2-dimyristyloxlpropyl-3-amine (PEG-C-DMA) (12)

PEG-C-DMA, compound **12**, was synthesized in a manner similar to PEG-C-DSA, compound **11**, on a 5 mmol scale to yield, after chromatography, compound **12** as a colourless solid (11.1 g, 88%, R_f=0.35 (8% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 5.21–5.15 (m, 1H, N*H*), 4.26–4.19 (m, 2H, C*H*$_2$OC(O)), 3.7–3.6 (m, ~180H, OC*H*$_{2(PEG)}$), 3.60–3.39 (m, 9H, NC*H*$_2$C*H*(OC*H*$_2$)CH$_2$OC*H*$_2$), 3.38 (s, ^{3}H, CH$_3$O), 1.60–1.50 (m, 4H, OCH$_2$C*H*$_{2(myristyl)}$), 1.38–1.20 (m, 44H, CH$_{2(myristyl)}$), 0.88 (t, 6H, CH$_2$C*H*$_3$, *J*=6.9 Hz).

3. Results

3.1. PEG-S-DSG is chemically unstable in liposomal formulations

To ascertain lipid stability, we studied lipid concentrations over time in SPLP samples stored at different temperatures. Formulations containing the lipids DSPC : cholesterol : PEG-S-DSG : DODMA (20:55:10:15 molar ratio) were examined by HPLC for degradation at 5, 25 and 40 °C. DODMA and cholesterol exhibited negligible structural instability, even under accelerated conditions (40 °C, Fig. 1A), while DSPC degraded to a certain extent. However, PEG-S-DSG degraded rapidly, with more than 10% of the lipid degrading in the first 2 months, even at 5 °C (Fig. 1B). As expected the instability of PEG-S-DSG was exacerbated at higher temperatures. The PEG-S-DSG half-life in aqueous liposomal formulations was 18.3, 5.2 and 2.7 months at 5, 25 and 40 °C, respectively. Half-life values were calculated using a first order, non-compartmental model. Log values of the concentrations were plotted against time to give straight lines, the slopes of which were used to calculate half-life.

3.2. Synthesis of novel PEG-lipids

Three novel PEG-lipids were synthesized for consideration as potential replacements for PEG-S-DSG; PEG-S-DSA, PEG-A-DSA and PEG-C-DSA. Each used a different linker to join the PEG to the lipid anchor (the previously described 1,2-distearyloxypropyl-3-amine (DSA) [38]); PEG-S-DSA, PEG-A-DSA and PEG-C-DSA utilized succinimide, amide and carbamate linkers, respectively (Fig. 2). The structure of DSA allows the possibility of synthesizing analogues with shorter alkyl chains, necessary for giving the PEG-lipids the desired programmable, exchangeable properties. The synthetic route to the lipids was comprehensively revised to allow more efficient manufacture, such that the overall yield was improved by approximately 50% while the number of synthetic steps was decreased from six to four. The synthesis and PEG-coupling processes (Fig. 3) displayed varying efficiency. Although the 2-step strategy used to obtain PEG-A-DSA had a low overall yield of 14%, this did not present a problem as the reactions are easily performed on large scale. PEG-S-DSA predictably had a low yield also (46%), having the longest synthetic route with 4 discrete steps. The PEG-C-DSA single-step synthesis was least complicated, and, unsurprisingly, had the highest yield of 90%. All synthetic products were
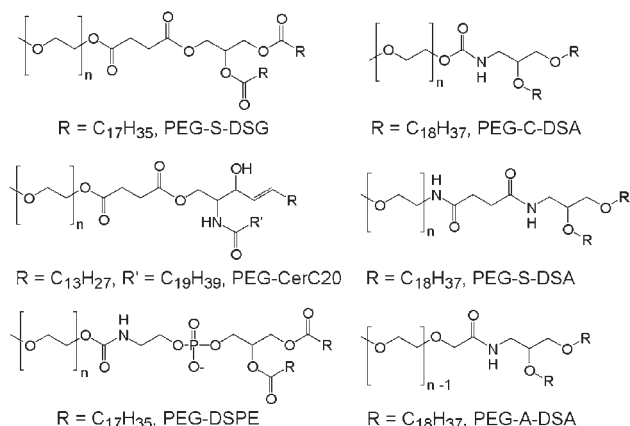
Fig. 2. Chemical structures of the 2000 molecular weight ($n=45$) PEG-lipids assessed as replacements for PEG-S-DSG (top left). The 3 novel lipids (right hand column) were designed without carboxylic ester bonds present in the readily available alternatives (left column).

analyzed by TLC and gave single spots with expected R_f values. They were further characterized by ^{1}H NMR and, in the case of non-polymeric compounds, elemental analysis.

3.3. Physical characterization of formulations

The formulation characteristics of the six PEG-lipids were evaluated with particular emphasis on particle size and encapsulation efficiency. The PEG-lipids were used to prepare SPLP by spontaneous vesicle formation [37]. The PEG-DSPE formulation had a mean particle size of 161 nm. All other formulations had the

Fig. 3. Synthesis of three novel PEG-lipids. The PEG-lipids possessed carbamate, amide and succinimide linkers, rather than the succinate linker used in PEG-S-DSG and PEG-CerC$_{20}$.

characteristic small particle size of SPLP (approximately 120 nm diameter, Table 1). Encapsulation efficiency was also significantly less with the use of PEG-DSPE; 61% as compared to 72% to 80% for the other 5 formulations ($P<0.05$, t-test). Final DNA encapsulation values were all somewhat similar, as the SPLP were passed through Mustang Q cartridges to remove unencapsulated DNA at the end of the formulation process. Even so, PEG-DSPE resulted in a formulation with a significantly lower percentage of encapsulated nucleic acid in the final product, as compared to the other PEG-lipids ($P<0.05$, t-test).

3.4. The presence of a succinate linker promotes instability in PEG-lipids

Assessment of PEG-lipid stability within the vesicles was of interest. Therefore, formulations containing the six PEG-lipids were analyzed for lipid degradation in an accelerated stability study, carried out over a six-week period at 37 °C. As expected from the earlier results, PEG-S-DSG was shown to degrade steadily over the course of the experiment ($t_{1/2}=58$ days) and PEG-CerC$_{20}$ degraded at a very similar rate ($t_{1/2}=47$ days) (Fig. 4). The breakdown of both lipids was shown to be significant, even after 7 days ($P<0.05$, t-test). The remaining PEG-lipids exhibited no obvious signs of instability during the six-week time-course, with concentrations after 42 days not significantly different from initial values ($P>0.1$ in all cases, t-test). The other lipids, cholesterol, DSPC and DODMA, were also stable during this time period. It is interesting to note that PEG-DSPE, despite containing two carboxylic ester bonds in its structure, appears to be stable within the context of this particular experiment.

3.5. In vivo gene expression from intravenous administration of SPLP

It has previously been shown that use of PEG-S-DSG or PEG-CerC$_{20}$ allows for the formation of long circulating particles that yield preferential gene expression in tumor tissue [8,9]. Ensuring a similar performance with the new PEG-lipids was of obvious importance. Therefore, gene expression was evaluated in a mouse tumor model following intravenous (i.v.) administration of SPLP containing the pCMVluc plasmid. 48 h

Table 1
Physical properties of the SPLP formulations

PEG-lipid	Mean particle diameter (nm±S.D.)	Initial encapsulated DNA (%)	Final encapsulated DNA (%)	Charge of PEG-lipid at formulation pH (5.0)
PEG-S-DSG	118±4	80±1	94±1	Neutral
PEG-DSPE	161±5	61±3	84±2	−1
PEG-CerC$_{20}$	109±6	74±2	94±2	Neutral
PEG-A-DSA	122±5	72±2	89±2	Neutral
PEG-S-DSA	122±6	75±1	95±1	Neutral
PEG-C-DSA	118±5	72±2	89±1	Neutral
PEG-C-DMA	122±5	78±2	95±2	Neutral

Values are the mean of 3 separate experiments, the error stated is the standard deviation.

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Fig. 4. Stability of PEG-lipids in SPLP at 40 °C. Lipid concentrations were determined by HPLC analysis. Error shown is standard deviation ($n=3$).

after SPLP administration, luciferase expression was evaluated in the liver, lung, spleen heart and tumor. As shown previously with PEG-S-DSG [9] or PEG-CerC$_{20}$ [40] containing SPLP, an excellent differential in luciferase protein levels was observed between the tumor and other tissues (Fig. 5). All six formulations were similar in this respect, typically resulting in tumor gene expression 2 orders of magnitude greater than that observed in other, non-target tissues.

3.6. Intravenous administration of SPLP is well tolerated

The relative toxicity of the new PEG-lipids was clearly of interest, as they are ultimately intended for in vivo applications. The toxicity following SPLP administration was examined by determining levels of the transaminases ALT and AST in mouse serum following SPLP treatment (Fig. 6). ALT is primarily regarded as a hepatocytes protein and increases in ALT are thought to indicate liver damage. AST is present in most tissues but particularly in cardiac muscle, skeletal muscle and the liver. Elevations in AST are regarded as a more general indication of systemic tissue damage. None of the formulations resulted in large increases in either enzyme, and ALT and AST levels actually remained within the normal limits for the PEG-A-DSA and PEG-

C-DSA SPLP. The remaining four formulations exhibited only marginally increased levels of either enzyme.

3.7. Circulation half-life of SPLP comprising PEG-lipids with shorter lipid anchors

Formulation, stability, transfection and toxicity data, combined with the ease of synthesis, lead to the selection of the carbamate linker chemistry for further characterization. The C$_{14}$ analogue (PEG-C-DMA) was synthesized to confirm that, similarly to PEG-S-DAGs and PEG-Ceramides, bilayer exchangeability (and thus pharmacology) could be modulated by varying the size of the lipid anchor. SPLP were prepared incorporating a ^{3}H-CHE-lipid marker. The percentage of injected dose remaining in circulation following a single injection of SPLP in the tail vein is displayed as a function of time (Fig. 7A). SPLP containing PEG-C-DSA exhibited a circulation half-life of 16 h, similar to that of the PEG-S-DSG containing formulation. SPLP containing PEG-C-DMA cleared more rapidly with a half-life of approximately 2 h. These results are very similar to those reported previously for SPLP containing the PEG-DAGs [9] and PEG-Ceramides [7]. The initial phase of the curve (from 0 to 8 h) was used to calculate half-life, using a first order, non-compartmental model. This initial phase 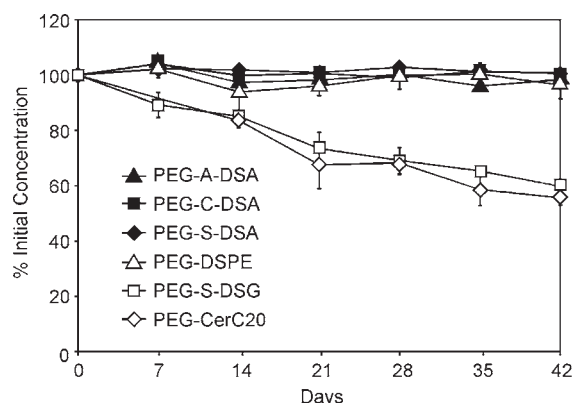was felt to be the most relevant part of the curve, as it indicates how quickly the majority of the SPLP dose is cleared. It also represents 4 out of the 5 data-points. Log values of the concentrations were plotted against time to give straight lines, the slopes of which were used to calculate half-life.

3.8. Gene expression patterns from formulations containing PEG-lipids with shorter lipid anchors

We have previously determined that the use of PEG-lipids with a shorter, C$_{14}$ lipid anchor is necessary to avoid the generation of an antibody response when delivering encapsulated nucleic acids [30,31]. The main drawback to this strategy is that the shorter lipid anchor leads to faster clearance, meaning less time for the

Fig. 5. Biodistribution of luciferase gene expression in Neuro-2a tumor-bearing male A/J mice. Gene expression was assessed 48 h after a single intravenous administration of SPLP. Error shown is standard deviation ($n=6$).

Fig. 6. Toxicity resulting from intravenous administration of SPLP. Serum was collected from mice and assayed for the enzymes alanine transferase (ALT) and aspartate transferase (AST). The upper and lower limits of historical normal values, as quoted by the Canadian Council on Animal Care, are indicated by dotted lines. Error bars represent standard deviation ($n=3$).

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Fig. 7. (A) Plasma clearance of SPLP containing PEG-S-DSG, PEG-C-DSA or PEG-C-DMA. The percentage of injected dose remaining in plasma of mice following a single intravenous administration is displayed. SPLP were labelled with ^{3}H-cholesteryl hexadecyl ether (1 µCi per mg of lipid). Error bars represent standard deviation ($n=4$). (B) Biodistribution of gene expression resulting from administration of SPLP containing C_{14} or C_{18} PEG-lipid analogues. SPLP were administered by intravenous administration and gene expression measured 48 h later. Error shown is standard deviation ($n=4$).

particles to accumulate at the disease site and typically resulting in lower gene expression at distal tumor sites. It was of interest to evaluate to what degree this phenomenon affected gene expression using PEG-C-DMA SPLP. SPLP were prepared using the C_{14} (PEG-C-DMA and PEG-succinoyl dimyristylglycerol (PEG-S-DMG)) and C_{18} (PEG-C-DSA and PEG-S-DSG) analogues of PEG-C-DAAs and PEG-S-DAGs. Gene expression was evaluated in the liver, lung, spleen heart and tumor 48 h after a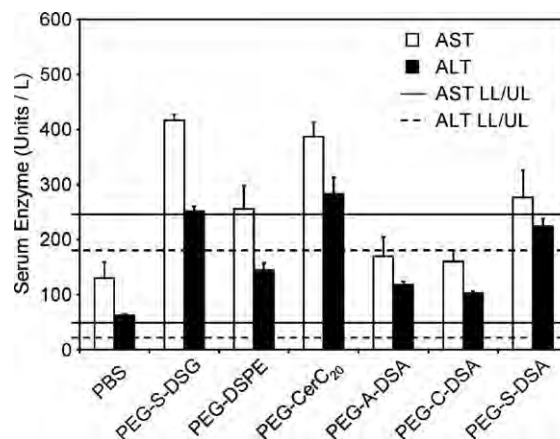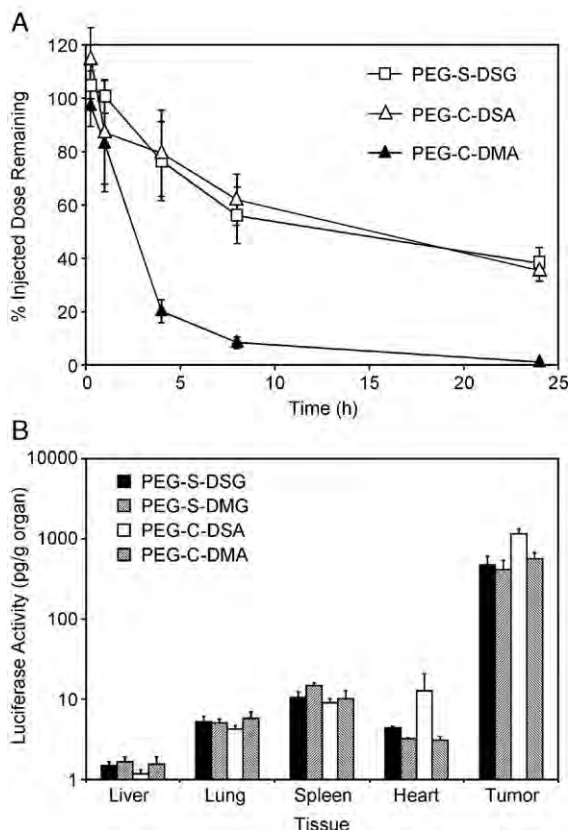 single intravenous administration of SPLP. Although tumor gene expression resulting from PEG-C-DMA SPLP treatment was significantly lower than that obtained with PEG-C-DSA ($P<0.05$), there was no significant difference between the level of tumor gene expression following treatment with PEG-S-DMG, PEG-S-DSG, or PEG-C-DMA SPLP (Fig. 7B). Consistent with previous results, a marked differential was seen between the gene expression in tumor tissue and the other tissues examined, typically of 2 orders of magnitude.

4. Discussion

While conducting long-term stability tests on SPLP, we discovered that the PEG-lipid, PEG-S-DSG, was unstable.

Upon storage, even at 4 °C, more than 10% of the PEG-lipid was degraded within 2 months (Fig. 1). This effectively disqualifies this PEG-lipid from incorporation into any formulation to be considered for clinical development. We hypothesized that this instability was due to the presence of readily hydrolysable carboxylic ester bonds (of which there are 4 in the structure of PEG-S-DSG) [41,42]. Of the possible replacements, PEG-Ceramides [21] and PEG-DSPE possess ester bonds. PEG-DSPE also possesses an electrostatic charge, at both formulation and physiological pH, as do SAINT-PEGs [23]. This was also considered to be undesirable, for reasons of increased blood clearance [43,44], possible complement activation, and/or possible leakage of encapsulated material [21].

Another strategy for facilitating the intracellular delivery of PEG-lipid-containing particles is the use of PEG-lipids designed to cleave in the endosome. These include the disulphide-, vinyl ether- and diorthoester-linked PEG-lipids mentioned previously [24–29]. However, when considering the PEGylation strategy for nucleic acid containing particles, it is important to consider recent reports of an acquired immune response to C_{18} PEG-lipids with a long residence time on the particle [30,31]. The environmentally sensitive PEG-lipids described to date utilize C_{18} lipid anchors, and therefore may illicit similar immune responses when used to deliver immunostimulatory nucleic acids. The use of PEG-lipid linker chemistries that were designed to cleave in response to environmental triggers was rejected in favor of using exchangeable PEG-lipids.

We designed three novel PEG-lipids as potential replacements for PEG-S-DSG, each containing a different linker connecting the PEG and lipid domains. The first, PEG-S-DSA, utilized a succinimide linker, a direct (amide) analogue of the labile succinate linker in PEG-S-DSG. The yield for coupling of the PEG and lipid moieties was quite low, with an overall yield of 14%. The second and third PEG-lipids, PEG-A-DSA and PEG-C-DSA, possessed simple amide and carbamate linkers. These required fewer steps to synthesize and yields were much higher, 46% and 90%, respectively. All novel PEG-lipids utilized the same type of lipid anchor. Similarly to the linker, the carboxylic esters in the lipid anchor were replaced, in this case with ether bonds. The lipid possesses a primary amine head group to facilitate coupling to PEG. The dialkylglyceryl nature of the hydrophobic anchor was retained, to facilitate straightforward adjustment of the diffusible nature of the resulting PEG-lipid conjugate. When performing PEG-lipid coupling, an excess of lipid was used in relation to PEG. While this may seem counterintuitive (since the lipid anchor requires more synthetic steps to prepare than PEG), subsequent work up and purification of the final compounds was found to be easier with this approach. The C_{18} analogues of all PEG-lipids were synthesized initially to allow for direct comparison with PEG-S-DSG.

All of the PEG-lipids, with the exception of PEG-DSPE, were readily incorporated in SPLP and resulted in acceptable formulations. PEG-DSPE, being the only PEG-lipid to possess a negative charge, yielded larger particles (~160 nm) than the

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five neutral compounds (~120 nm). The model for SPLP formation and nucleic acid encapsulation involves a charge interaction between nascent cationic lipid-containing bilayer fragments and the negatively charged nucleic acid. PEG-DSPE may act to partially neutralize the charge on the bilayer fragments or actively repel the DNA by charge repulsion, as encapsulation efficiency was also reduced when using PEG-DSPE.

Stability assessment of the individual PEG-lipids incorporated in SPLP yielded interesting results (Fig. 4). PEG-S-DSG was shown to degrade steadily in aqueous solution, as was the PEG-CerC$_{20}$. PEG-DSPE, however, is stable, despite possessing two carboxylic ester bonds similar to those thought to contribute to PEG-S-DSG instability. This may be explained by the location of the ester bonds. PEG-S-DSG and PEG-CerC$_{20}$ both contain ester bonds in the succinate linkers, between the PEG and hydrophobic domains of the molecule. These are expected to be located at the surface of the SPLP lipid bilayer, allowing ingress of water molecules necessary for the hydrolysis of this bond. Conversely, the ester bonds of PEG-DSPE are located in the hydrophobic domain of the molecule and would be expected to be sequestered deep within the lipid bilayer. These bonds would be less accessible to water and therefore less susceptible to hydrolysis. Of note, DSPC, which also contains ester bonds in the hydrophobic domain, also appears to be relatively stable within the limited context of this experiment. The three novel PEG-lipids, containing no ester bonds, were completely stable, as were the cholesterol and DODMA components.

To assess the utility of the PEG-lipids in formulations for systemic delivery, SPLP containing a luciferase reporter plasmid were administered to Neuro-2a tumor-bearing mice via tail vein injection. Since all the PEG-lipids used in this experiment were C$_{18}$ analogues, and the hydrophobic domain of the molecule is the dominant factor in determining transfection efficiency, it was not unexpected that the six formulations performed similarly. Each formulation resulted in comparable luciferase expression of approximately 700 pg/g of tumor. This compares favorably with previously reported SPLP formulations containing pCMVluc plasmids, which have yielded 30 [7] and 100 pg/g [9]. The preferential expression of luciferase in the tumor is in part due to passive disease site targeting and the EPR effect, but also because non-viral delivery systems transfect actively dividing cells more efficiently [45].

Analysis of serum transaminase levels confirmed comparable, low toxicity for all systems. AST and ALT levels were elevated only slightly in mice treated with 50 mg/kg total lipid, of which approximately 20 mg/kg was PEG-lipid, when compared to PBS controls. This was consistent with previous results in which SPLP have been shown to be less toxic than lipoplex systems [7]. The move from readily degradable ester PEG-lipids, to those with more stable, and possibly less easily metabolised bonds, had no apparent effect on acute toxicity.

The data favored the adoption of PEG-C-DSA or one of its analogues. PEG-C-DSA is stable in the bilayer at 40 °C for greater than six weeks, it is charge-neutral, formulates well, is

synthesized in good yield with a minimum number of steps, and gives particles that transfect as well as PEG-S-DSG without signs of toxicity. To confirm the diffusible PEG-lipid paradigm would apply to PEG-C-DSA analogues and to address the issue of the immune response with more stably incorporated PEG-lipids, the C$_{14}$ analogue (PEG-C-DMA) of PEG-C-DSA was synthesized. The synthesis and purification of PEG-C-DMA, like its C$_{18}$ analogue, were found to be straightforward and of high yield (88% compared to 90%). PEG-C-DMA particles were found to possess the same small size and high encapsulation characteristics as those containing PEG-C-DSA (Table 1). The pharmacokinetics of PEG-C-DMA-containing SPLP was confirmed in PK studies utilizing ^{3}H-labelled SPLP. As with PEG-S-DSG/PEG-S-DMG formulations, where long ($t_{1/2}=15$ h) and short ($t_{1/2}=1$ h) circulating formulations were prepared by switching from the C$_{18}$ to a C$_{14}$ lipid anchor [9], the circulation half-life of PEG-C-DSA ($t_{1/2}=16$ h) and PEG-C-DMA ($t_{1/2}=2$ h) supported the diffusible PEG-lipid paradigm. Of interest, although the reduced circulation time had an effect on the resulting transfection efficiency (Fig. 7B), the tumor gene expression resulting from the administration of PEG-C-DMA-containing SPLP was as great as that obtained when using PEG-S-DSG suggesting that PEG-C-DMA may be a suitable replacement for PEG-S-DSG in nucleic acid delivery systems. Subsequently, toxicity evaluation by analysis of serum transferase levels was performed, following intravenous administration of PEG-C-DMA SPLP in A/J mice (data not shown). Similarly to the PEG-C-DSA analogue, AST and ALT levels of 125 and 78 IU/L, respectively, were barely elevated, and well within normal limits as set out by the Canadian Council on Animal Care (CCAC).

5. Conclusion

Using a simple assay developed to determine lipid degradation, we have demonstrated that two of the better-known types of PEG-lipid, PEG-S-DAGs and PEG-Ceramides, are unstable when incorporated in aqueous liposomal formulations. Accordingly, we have designed and synthesized three replacement PEG-lipids. We have shown that all three of the novel PEG-lipids are readily synthesized, stable under stressed conditions and formulate well as SPLP. The resulting particles are non-toxic and capable of transfecting distal tumors with 2 orders of magnitude of specificity over other organs. Our continuing changes to the lipid components of the SPLP particle, with no obvious penalty in terms of formulability or performance, further demonstrate the robustness of the SPLP platform and formulation methodology.

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JOINT APPENDIX 44

PCT

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(54) Title: HIGH EFFICIENCY ENCAPSULATION OF CHARGED THERAPEUTIC AGENTS IN LIPID VESICLES

(57) Abstract

Methods for the preparation of a lipid–nucleic acid composition are provided. According to the methods, a mixture of lipids containing a protonatable or deprotonatable lipid, for example an amino lipid and a lipid such as a PEG– or polyamide oligomer–modified lipid is combined with a buffered aqueous solution of a charged therapeutic agent, for example polyanionic nucleic acids, to produce particles in which the therapeutic agent is encapsulated in a lipid vesicle. Surface charges on the lipid particles are at least partially neutralized to provide surface–neutralized lipid–encapsulated compositions of the therapeutic agents. The method permits the preparation of compositions with high ratios of therapeutic agent to lipid and with encapsulation efficiencies in excess of 50 %.

pH 4.0 INTERNAL

pH 4.0 EXTERNAL

COLUMN CHROMATOGRAPHY

1. EXCHANGE pH 4.0 CITRATE FOR pH 7.5 HBS
2. NEUTRALIZE SURFACE DODAP; ANTISENSE RELEASE
3. REMOVAL OF NON–ENCAPSULATED ANTISENSE

RELEASE OF SURFACE ANTISENSE

pH 4.0 INTERNAL

pH 7.5 EXTERNAL

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BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
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CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

- 1 -

HIGH EFFICIENCY ENCAPSULATION OF CHARGED
THERAPEUTIC AGENTS IN LIPID VESICLES

DESCRIPTION

FIELD OF THE INVENTION

This invention relates to compositions comprising a combination of a lipid and a therapeutic agent, particularly to lipid-nucleic acid compositions, for *in vivo* therapeutic use. In these compositions the therapeutic agent is encapsulated and protected from degradation

5   and clearance in serum. Additionally, the invention provides methods of making the compositions, as well as methods of introducing the nucleic acids into cells using the compositions and treating disease conditions.

BACKGROUND OF THE INVENTION

10   Therapeutic oligonucleotides, such as antisense oligonucleotides or ribozymes, are short segments of DNA that have been designed to hybridize to a sequence on a specific mRNA. The resulting complex can down-regulate protein production by several mechanisms, including inhibition of mRNA translation into protein and/or by enhancement of RNase H degradation of the mRNA transcripts. Consequently, therapeutic oligonucleotides

15   have tremendous potential for specificity of action (i.e. the down-regulation of a specific disease-related protein). To date, these compounds have shown promise in several *in vitro* and *in vivo* models, including models of inflammatory disease, cancer, and HIV (reviewed in Agrawal, *Trends in Biotech.* 14:376-387 (1996)). Antisense can also effect cellular activity by hybridizing specifically with chromosomal DNA. Advanced human clinical assessments of

20   several antisense drugs are currently underway. Targets for these drugs include the genes or RNA products of c-myc, ICAM-1, and infectious disease organisms such as cytomegalovirus, and HIV-1.

One well known problem with the use of therapeutic oligonucleotides having a phosphodiester internucleotide linkage is its very short half-life in the presence of serum or

25   within cells. (Zelphati, O et al. 1993. Inhibition of HIV-1 Replication in Cultured Cells with Antisense Oligonucleotides Encapsulated in Immunoliposomes. Antisense. Res. Dev. 3:323-338; and Thierry, AR et al. pp147-161 in Gene Regulation: Biology of Antisense RNA and

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DNA (Eds. Erickson, RP and Izant, JG) 1992. Raven Press, NY). No clinical assessment currently employs the basic phosphodiester chemistry found in natural nucleic acids, because of these and other known problems.

This problem has been partially overcome by chemical modifications which
5   reduce serum or intracellular degradation. Modifications have been tested at the internucleotide phosphodiester bridge (i.e. using phosphorthioate, methylphosphonate or phosphoramidate linkages), at the nucleotide base (i.e.5-propynyl-pyrimidines), or at the sugar (i.e. 2'-modified sugars) (Uhlmann E., et al. 1997. Antisense: Chemical Modifications. Encyclopedia of Cancer Vol. X. pp 64-81 Academic Press Inc.). Others have
10 attempted to improve stability using 2'-5' sugar linkages (see US Pat. No. 5,532,130). Other changes have been attempted. However, none of these solutions have proven entirely satisfactory, and *in vivo* free antisense still has only limited efficacy. Problems remain, such as in the limited ability of some antisense to cross cellular membranes (see, Vlassov, *et al., Biochim. Biophys. Acta* **1197**:95-1082 (1994)) and in the problems associated with systemic
15 toxicity, such as complement-mediated anaphylaxis, altered coagulatory properties, and cytopenia (Galbraith, *et al., Antisense Nucl. Acid Drug Des.* 4:201-206 (1994)). Further, as disclosed in US Pat. Appl. SN. 08/657,753 and counterpart patent application WO 97/46671, both incorporated herein by reference, modified antisense is still highly charged, and clearance from the circulation still takes place within minutes.

20 To attempt to improve efficacy, investigators have also employed lipid-based carrier systems to deliver chemically modified or unmodified antisense. In Zelphati, O and Szoka, F.C. (1996) J. Contr. Rel. 41:99-119, the authors refer to the use of anionic (conventional) liposomes, pH sensitive liposomes, immunoliposomes, fusogenic liposomes and cationic lipid/antisense aggregates.

25 None of these compositions successfully deliver phosphodiester antisense for *in vivo* therapy. In another paper, Zelphati & Szoka note that antisense phosphodiester oligonucleotides associated with cationic lipids have not been active in cell culture *in vitro*; and that only one study has reported the activity of phosphodiester antisense oligonucleotides complexed to cationic lipids. The authors argue that these findings "...necessitate[ ] the use
30 [of – sic] backbone-modified oligonucleotides that are relatively resistant to both intracellular and extracellular nucleases even if a carrier is used to deliver the oligonucleotide into the

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target cell". (1997. J. Lip. Res. 7(1):31-49 at 34). This finding is corroborated by Bennett, CF. (1995. Intracellular Delivery of Oligonucleotides with Cationic Liposomes. Chp 14 CRC Press) who states at p. 224 that "In contrast, we have been unable to demonstrate inhibition of gene expression by uniform phosphodiester oligodeoxynucleotides directed

5   towards a number of cellular targets in the presence of cationic lipids."

Prior art lipid formulations of <u>modified antisense</u> are also largely ineffective *in vivo*. They have poor encapsulation efficiency (15% or less for passive encapsulation systems), poor drug to lipid ratios (3% or less by weight), high susceptibility to serum nucleases and rapid clearance from circulation (particularly in the case of cationic

10   lipid/antisense aggregates made from DOTMA, trade-name LIPOFECTIN™), and/or large sized particles (greater than 100 nm), which make them unsuitable for systemic delivery to target sites. No successful *in vivo* efficacy studies of lipid-encapsulated (nuclease-resistant) modified antisense are known in the prior art.

Two references to unique lipid-antisense compositions that may be

15   significantly nuclease resistant bear consideration. Firstly, the anionic liposome (LPDII) composition of Li, S. and Huang, L (1997. J. Lip. Res. 7(1) 63-75), which encapsulates poly-lysine coated antisense, are said to have 60-70% encapsulation efficiency, but suffer from a large size of around 200 nm and a low drug to lipid ratio of 8% by weight. The effect of these particles *in vivo* is unknown. Secondly, the Minimal Volume Entrappment (MVE)

20   technique for cardiolipin (anionic) liposomes results in the reasonably high encapsulation efficiency of 45-65% but again the drug:lipid ratio remains very small, approximately 6.5% by weight (see US Pat. No. 5,665,710 to Rahman et al.; Thierry AR, and Takle, GB. 1995, Liposomes as a Delivery System for Antisense and Ribozyme Compounds. in *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, S. Akhtar, ed, CRC Press, Boca

25   Raton, FL., pp. 199-221; Thierry, AR et al. pp147-161 in Gene Regulation: Biology of Antisense RNA and DNA (Eds. Erickson, RP and Izant, JG) 1992. Raven Press, NY). Note that US Pat. No. 5,665,710 also discloses encapsulation efficiencies of 60-90% for tiny, medically useless amounts of antisense (0.1 ug), where the drug to lipid ratio must be very low.

30   It is an observation of the inventors that a wide variety of prior art lipid compositions used for conventional drugs could be tested for efficacy in the antisense field,

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but the improvement (over free antisense) for *in vivo* efficacy is not known. In this regard, it is noted that although lipid compositions assertedly for use as drug carriers were disclosed by Bailey and Cullis (US Pat. 5552155; and (1994) Biochem. 33(42):12573-12580), they did not disclose formulations of any bioactive compounds with these lipids, and did not suggest their

5   utility for high efficiency loading of polyanionic species.

What is needed in the art are improved lipid-therapeutic oligonucleotide compositions which are suitable for therapeutic use. Preferably these compositions would encapsulate nucleic acids with high-efficiency, have high drug:lipid ratios, be encapsulated and protected from degradation and clearance in serum, and/or be suitable for systemic

10   delivery. The present invention provides such compositions, methods of making the compositions and methods of introducing nucleic acids into cells using the compositions and methods of treating diseases.

SUMMARY OF THE INVENTION

15   In accordance with the invention, charged therapeutic agents are packaged into lipid-encapsulated therapeutic agent particles using a method comprising the steps of:

(a)   combining a mixture of lipids comprising at least a first lipid component and a second lipid component with a buffered aqueous solution of a charged therapeutic agent to form an intermediate mixture containing lipid-encapsulated therapeutic

20   particles, and

(b) changing the pH of the intermediate mixture to neutralize at least some exterior surface charges on said lipid-nucleic acid particles to provide at least partially-surface neutralized lipid-encapsulated therapeutic agent particles. The first lipid component is selected from among lipids containing a protonatable or deprotonatable group that has a

25   pKa such that the lipid is in a charged form at a first pH and a neutral form at a second pH, preferably near physiological pH. The buffered solution has a pH such that the first lipid component is in its charged form when in the buffered solution, and the first lipid component is further selected such that the charged form is cationic when the therapeutic agent is anionic in the buffered solution and anionic when the therapeutic agent is cationic in the buffered

30   solution. The second lipid component being selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation. The method the invention is

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particularly useful for preparation of lipid-encapsulated nucleic acids, for example antisense nucleic acids or ribozyme.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a neutralization step which releases surface-bound antisense from the lipid-nucleic acid compositions according to the present invention.

Figures 2A and 2B illustrate certain lipid components which are useful in the present inventive methods. Figure 2A illustrates several groups of amino lipids including the chemical structure of DODAP. Figure 2B illustrates groups of PEG-modified lipids.

Figure 3 illustrates the influence of ethanol on the encapsulation of antisense oligodeoxynucleotides. The liposomal antisense compositions were prepared as described in the Examples, with the final concentrations of antisense and lipids being 2 mg/mL and 9.9 mg/mL, respectively. The final ethanol concentration in the preparations was varied between 0 and 60%, vol/vol. Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

Figure 4 illustrates the influence of ethanol on lipid and antisense loss during extrusion. The liposomal antisense compositions were prepared as described for Figure 3. The samples were extruded ten times through three 100 nm filters as described in "Materials and Methods". After extrusion, the filters were analyzed for [^{3}H]-antisense and [^{14}C]-lipid radioactivity by standard scintillation counting techniques. Results were expressed as a percent of the total initial radioactivity.

Figure 5 illustrates the influence of DODAP content on the encapsulation of antisense oligodeoxynucleotides. A 0.6 mL aliquot of a [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with 0.4 mL of a 95% ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 100-(55+X):45:X:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The molar ratio of DODAP was varied between 0 and 30%. The molar ratio of DSPC was adjusted to compensate for the changes in DODAP content. Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by

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determining the total pre-column and post-column [³H]-antisense and [¹⁴C]-lipid radioactivity.

     Figure 6 illustrates the influence of DODAP content on the encapsulation of antisense oligodeoxynucleotides. Samples were identical to those prepared in Figure 5. In
5   this instance, the amount of antisense associated with the lipid was assessed by a solvent extraction procedure as described in "Material and Methods". Antisense was extracted into a methanol:water aqueous phase, while the lipid was soluble in the organic (chloroform) phase. The aqueous phase was preserved and antisense concentration was determined by measuring the absorbance at 260 nm. This confirmed that the antisense was associated with the lipid
10   vesicles, and that the [³H]-label on the antisense had not exchanged to the lipid.

     Figure 7 illustrates the quasi-elastic light scattering analysis of encapsulated liposomal antisense. The size distribution of a liposomal preparation of antisense was determined by quasi-elastic light scattering (QELS) immediately after removal of the free antisense (A), and after storage of the preparation for 2 months at 4°C (B), using a Nicomp
15   Model 370 sub-micron particle sizer.

     Figure 8 illustrates the influence of the initial antisense concentration on antisense loading in DODAP vesicles. Varying final concentrations of a 20mer of [³H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) were mixed with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14;
20   25:45:20:10, molar ratio), 9.9 mg/mL (final concentration). Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [³H]-antisense and [¹⁴C]-lipid or by determining the total pre-column and post-column [³H]-antisense and [¹⁴C]-lipid radioactivity. EPC:CHOL liposomes containing encapsulated antisense are included for comparison.

25   **Figure 9** illustrates the plasma clearance of encapsulated antisense. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP:PEG-CerC14 (25:45:20:10), where X represents either distearoylphosphatidylcholine (DSPC),
30   sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ([¹⁴C]-cholesterylhexadecylether) and [³H]-antisense and were injected

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(200 µL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

Figure 10 illustrates the biodistribution of encapsulated antisense.

5   Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP: PEG-CerC14 (25:45:20:10), where X represents either distearoyl-phosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The

10   formulations contained a lipid label ($[^{14}C]$-cholesterylhexadecylether) and $[^3H]$-antisense and were injected (200 µL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Mice were terminated by cervical dislocation and the organs were recovered and processed as described in "Materials and Methods". Lipid and antisense recoveries were determined by standard scintillation counting techniques.

15   **Figure 11** illustrates the differential release rates of antisense in plasma. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP: PEG-CerC14 (25:45:20:10), where X represents either distearoyl-phosphatidylcholine

20   (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ($[^{14}C]$-cholesterylhexadecylether) and $[^3H]$-antisense and were injected (200 µL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

25   Release rates were determined by measuring the [3H]/[14C] ratio over time.

Figure 12 illustrates the influence of PEG-acyl chain lengths on plasma clearance of encapsulated antisense. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were

30   composed of DSPC:CHOL:DODAP:PEG-CerC14 or C20 (25:45:20:10). The formulation contained a lipid label ($[^{14}C]$-cholesterylhexadecylether) and $[^3H]$-antisense and were injected

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(200 µL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

Figure 13 illustrates the enhanced efficacy of liposomal antisense containing
5   DODAP - ear swelling. Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped PS 3082 (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped PS 3082 (identified as 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped PS 3082 (identified as 4200). Ear swelling was measured at 24 hours after initiating inflammation
10   using an engineer's micrometer.

Figure 14 illustrates the enhanced efficacy of liposomal antisense containing DODAP - cellular infiltration. Mice received 10 µCi of [^{3}H]-methylthymidine, i.p., 24 hours before initiating inflammation. Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped PS 3082
15   (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped PS 3082 (identified as 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped PS 3082 (identified as 4200). Cell infiltration was monitored by measuring the radioactivity in the "challenged ear" versus the non-treated ear. Results are expressed as the ratio of radioactivity in the left (challenged ear) versus right ear.

20   **Figure 15** shows asymmetric loading of lipid-encapsulated-nucleic acid particles in accordance with the invention .

Figure 16 shows clearance of lipid-encapsulated antisense particles formulated with several amino lipids at different levels.

Figure 17 shows blood levels of antisense-containing particles after repeat
25   dosages.

Figure 18 shows blood levels of antisense-containing particles after repeat dosages.

Figure 19 illustrates results of a study on the *in vivo* efficacy of lipid-encapsulated antisense particles in accordance with the invention in a mouse tumor model.
30   **Figure 20** shows encapsulation efficiency results for lipid-encapsulated therapeutic agent particles in accordance with the invention.

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Figure 21 shows results for studies on the use of murine ICAM1 in an ear inflammation model.

Figure 22 shows results for studies on the use of murine ICAM1 in an ear inflammation model.

5   **Figure 23** shows results for studies on the use of murine ICAM1 in an ear inflammation model.

DETAILED DESCRIPTION OF THE INVENTION
CONTENTS

I.   **Glossary**

Abbreviations and Definitions

20   The following abbreviations are used herein: ATTA, N-(ω-N'-acetoxy-octa(14'amino-3',6',9',12'-tetraoxatetradecanoyl)); CHE, cholesteryl-hexadecylether; CHOL, cholesterol; DODAP or AL-1, 1,2-dioleoyloxy-3-dimethylaminopropane (and its pro-tonated ammonium form); DODMA, N-(1-(2,3-Dioleoyloxy) propyl)-N,N,-dimethyl ammonium chloride; DSPC, distearoylphosphatidylcholine; EPC, egg phosphatidylcholine; 25   HBS, HEPES-buffered saline; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; MES, 2-(N-morpholino)ethane sulfonic acid; PS 3082, murine ICAM-1 phosphoro-thioate oligodeoxynucleotide having the sequence: TGCATCCCCCAGGCCACCAT (SEQ ID No. 1); NaCl, sodium chloride; OLIGREEN™, a dye that becomes fluorescent when interacting with an oligonucleotide; PEG-CerC20, polyethylene glycol coupled to a ceramide 30   derivative with 20 carbon acyl chain; POPC, palmitoyloleoylphophatidylcholine; SM, sphingomyelin.

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"Lipid-therapeutic agent particle" means a particle comprising lipids and a charged (cationic or anionic) therapeutic agent.   "Lipid-therapeutic nucleic acid particle" means a particle comprising a lipid and a therapeutic nucleic acid.

"Lipid-encapsulated therapeutic agent (nucleic acid) particle" means a lipid-
5   therapeutic agent particle wherein less than 50% and preferably less than 10% of the therapeutic agent (nucleic acid) is detectable on the external surface of the particle or in the buffer external to the particle. In the case of nucleic acids, the amount of encapsulated versus unencapsulated nucleic acid can be assayed by fluorescence assays or nuclease assays as described herein. Comparable assays can be used for other types of therapeutic agents.
10   "Therapeutically effective amount" means an amount which provides a therapeutic benefit.   For antisense oligonucleotide this means generally 0.5 to 50 mg/kg of body weight, but when delivered in a lipid particle formulation, a below-toxic amount of lipid must be used.

"Lipid exchange out of particle" and the rate of this exchange is fully
15   explained in US Pat. Apps. SN 08/486,214 and 08/485,608 and PCT Patent publications WO 96/10391 and WO 96/10392, which are all incorporated herein by reference. Lipid exchange into the surrounding medium is possible for lipids which are reversibly associated with the lipid particle membrane. Each lipid has a characteristic rate at which it will exchange put of a particle which depends on a variety of factors including acyl chain length, saturation, head
20   group size, buffer composition and membrane composition.

"Disease site" is the site in an organism which demonstrates or is the source of a pathology. The disease site may be focused, as in a site of neoplasm or inflammation, or may be diffuse as in the case of a non-solid tumor. "Administration at a site which is distal to the disease site" means that delivery to the disease site will require some kind of systemic
25   delivery, either by blood or lymph circulation, or other fluid movement inside the organism.

The term "transfection" as used herein, refers to the introduction of polyanionic materials, particularly nucleic acids, into cells. The polyanionic materials can be in the form of DNA or RNA which is linked to expression vectors to facilitate gene expression after entry into the cell. Thus the polyanionic material or nucleic acids used in the
30   present invention is meant to include DNA having coding sequences for structural proteins, receptors and hormones, as well as transcriptional and translational regulatory elements (*i.e.,*

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promoters, enhancers, terminators and signal sequences) and vector sequences. Methods of incorporating particular nucleic acids into expression vectors are well known to those of skill in the art, but are described in detail in, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) or *Current*

5   *Protocols in Molecular Biology*, F. Ausubel *et al.*, ed. Greene Publishing and Wiley-Interscience, New York (1987), both of which are incorporated herein by reference.

The term "physiological pH" refers to pH levels conventionally encountered in serum or blood. In general, this will be in the range of pH 7.2 to 7.5. Preferred protonatable or deprotonatable lipids have a pKa such that they are substantially neutral at this pH, i.e., a

10   pKa of about 4 to 7 in the case of an amino lipid.

II.   General

The present invention relates to methods and compositions for producing lipid-encapsulated therapeutic agent particles in which charged therapeutic agents are

15   encapsulated within a lipid layer. The invention is applicable to both anionic and cationic therapeutic agents, including polyanionic nucleic acids, polyanionic proteins or peptides, cytokines and heparin, and cationic proteins and peptides. The invention is principally demonstrated herein with reference to polyanionic nucleic acids as the therapeutic agent, which is a preferred embodiment, but the same principles can be readily extended to other

20   polyanionic or to cationic therapeutic agents.

To evaluate the quality of a lipid/nucleic acid formulation the following criteria, among others, may be employed:

drug to lipid ratio;

encapsulation efficiency;

25   nuclease resistance/serum stability; and

particle size.

High drug to lipid rations, high encapsulation efficiciency, good nuclease resistance and serum stability and controllable particle size, generally less than 200 nm in diameter are desirable In addition, the nature of the nucleic acid polymer is of significance, since the

30   modification of nucleic acids in an effort to impart nuclease resistance adds to the cost of therapeutics while in many cases providing only limited resistance. The present invention

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provides lipid-nucleic acid particles and methods for preparing lipid-nucleic acid formulations which are far superior to the art according to these criteria.

Unless stated otherwise, these criteria are calculated in this specification as follows:

5   drug to lipid ratio: The amount of drug (therapeutic agent) in a defined volume of preparation divided by the amount of lipid in the same volume. This may be on a mole per mole basis or on a weight per weight basis, or on a weight per mole basis. For final, administration-ready formulations, the drug:lipid ratio is calculated after dialysis, chromatography and/or enzyme (e.g., nuclease) digestion has been employed to remove as

10   much of the external therapeutic agent (e.g., nucleic acid) as possible. Drug:lipid ratio is a measure of potency of the formulation, although the highest possible drug:lipid ratio is not always the most potent formulation;

encapsulation efficiency: the drug to lipid ratio of the starting mixture divided by the drug to lipid ratio of the final, administration competent formulation. This is a

15   measure of relative efficiency. For a measure of absolute efficiency, the total amount of therapeutic agent (nucleic acid) added to the starting mixture that ends up in the administration competent formulation, can also be calculated. The amount of lipid lost during the formulation process may also be calculated. Efficiency is a measure of the wastage and expense of the formulation;

20   nuclease resistance/serum stability: the ability of the formulation to protect the nucleic acid therapeutic agents from nuclease digestion either in an *in vitro* assay, or in circulation. Several standard assays are detailed in this specification. Encapsulated particles have much greater nuclease resistance and serum stability than lipid-antisense aggregates such as DOTMA/DOPE (LIPOFECTIN™) formulations; and

25   size: the size of the particles formed. Size distribution may be determined using quasi-elastic light scattering (QELS) on a Nicomp Model 370 sub-micron particle sizer. Particles under 200 nm are preferred for distribution to neo-vascularized (leaky) tissues, such as neoplasms and sites of inflammation.

The methods and composition of the invention make use of certain lipids

30   which can be present in both a charged and an uncharged form. For example, amino lipids which are charged at a pH below the pK_a of the amino group and substantially neutral at a pH

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above the pK$_a$ can be used in a two-step process. First, lipid vesicles can be formed at the lower pH with (cationic) amino lipids and other vesicle components in the presence of nucleic acids. In this manner the vesicles will encapsulate and entrap the nucleic acids. Second, the surface charge of the newly formed vesicles can be neutralized by increasing the pH of the

5   medium to a level above the pK$_a$ of the amino lipids present, i.e., to physiological pH or higher. Particularly advantageous aspects of this process include both the facile removal of any surface adsorbed nucleic acid and a resultant nucleic acid delivery vehicle which has a neutral surface. Liposomes or lipid particles having a neutral surface are expected to avoid rapid clearance from circulation and to avoid certain toxicities which are associated with

10   cationic liposome preparations.

It is further noted that the vesicles formed in this manner provide formulations of uniform vesicle size with high content of nucleic acids. Additionally, the vesicles are not aggregate complexes, but rather are large unilamellar vesicles having a size range of from about 70 to about 200 nm, more preferably about 90 to about 130 nm.

15   Without intending to be bound by any particular theory, it is believed that the very high efficiency of nucleic acid encapsulation is a result of electrostatic interaction at low pH. Figure 1 provides an illustration of the processes described herein. More particularly, this figure illustrates a lipid-nucleic acid composition of amino lipids and PEG-modified lipids having encapsulated antisense nucleic acid and surface-bound antisense nucleic acid.

20   At acidic pH (shown as pH 4.0), the surface is charged and binds a portion of the antisense through electrostatic interactions. When the external acidic buffer is exchanged for a more neutral (pH 7.5, HBS) buffer, the surface of the lipid particle or liposome is neutralized, resulting in release of the antisense nucleic acid.

Encapsulation efficiency results in Figs. 15 show a further unexpected benefit

25   of the invention. As shown in the figure, for both phosphorothioate (PS-2302) and phosphodiester (PO-2302) formulations it is possible to obtain encapsulation efficiencies – i.e., the amount of nucleic acid that ends up on the inside of the particle – that are greater than 50%. Phosphodiesters achieve well over 60%, and phosphorothioates can be at least up to 80% encapsulated. The assymetry of loading is surprising, given that in the simplest model

30   of loading large unilamellar vesicles (LUV's) the therapeutic agent (nucleic acid) would be equally likely to associate with cationic charges on the inside and outside of the particle. A

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1:1 distribution (inside to outside) would suggest that the 50% on the outside should be removed upon neutralization of the outside surface charges, such that 50% efficiency would be the theoretical upper limit.   Through some unclear mechanism, however, the invention surprisingly provides an active process whereby the majority of the therapeutic agent (nucleic

5   acid) ends up protected on the inside of the particles.

III.   Methods of Preparing Lipid/Therapeutic Agent (Nucleic Acid) Formulations

In view of the above, the present invention provides methods of preparing lipid/nucleic acid formulations. In the methods described herein, a mixture of lipids is

10   combined with a buffered aqueous solution of nucleic acid to produce an intermediate mixture containing nucleic acid encapsulated in lipid particles wherein the encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 10 wt% to about 20 wt%. The intermediate mixture may optionally be sized to obtain lipid-encapsulated nucleic acid particles wherein the lipid portions are large unilamellar vesicles, preferably having a

15   diameter of 70 to 200 nm, more preferably about 90 to 130 nm.   The pH is then raised to neutralize at least a portion of the surface charges on the lipid-nucleic acid particles, thus providing an at least partially surface-neutralized lipid-encapsulated nucleic acid composition.

The mixture of lipids includes at least two lipid components: a first lipid

20   component that is selected from among lipids which have a pKa such that the lipid is cationic at pH below the pKa and neutral at pH above the pKa, and a second lipid component that is selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation.

The first lipid component of is a lipid (or a mixture of lipid species with

25   similar properties) which has at least one protonatable or deprotonatable group, such that the lipid is charged at a first pH (cationic or anionic, depending on the nature and pKa of the protonatable or deprotonatable group), and neutral at physiological pH. It will of course be understood that the addition or removal of protons as a function of pH is an equilibrium process, and that the reference to a charged or a neutral lipid refers to the nature of the

30   predominant species and does not require that all of the lipid be present in the charged or neutral form.   Lipids which have more than one protonatable or deprotonatable group, or

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which are zwitterrionic are not excluded from use in the invention. Protonatable lipids are particularly useful as the first lipid component of the invention when the pKa of the protonatable group is in the range of about 4 to about 11. Most preferred is pKa of about 4 to about 7, because these lipids will be cationic at the lower pH formulation stage, while

5   particles will be largely (though not completely) surface neutralized at physiological pH around pH 7.5. One of the benefits of this pKa is that at least some antisense stuck to the outside surface of the particle will lose its electrostatic interaction at physiological pH and be removed by simple dialysis; thus greatly reducing the particle's susceptibility to clearance.

         Preferred lipids with a protonatable group for use as the first lipid component

10   of the lipid mixture are amino lipids. As used herein, the term "amino lipid" is meant to include those lipids having one or two fatty acid or fatty alkyl chains and an amino head group (including an alkylamino or dialkylamino group) which is protonated to form a cationic lipid at physiological pH (see Figure 2A). In one group of embodiments, the amino lipid is a primary, secondary or tertiary amine represented by the formula:

15

$$R^1_{}\!\!\diagdown_{}\!\!\!\underset{R^2\diagup}{}N-R^3$$

in which R^1 is a C_{12} to C_{24} alkyl group which is branched or unbranched, and saturated or unsaturated. R^2 is hydrogen or a C_1 to C_{24} alkyl group which is also branched or unbranched, and saturated or unsaturated (when three or more carbons are present). R^3 is hydrogen or a C_1 to C_6 alkyl group. Examples of these amino lipids include, for example, stearylamine,

20   oleylamine, dioleylamine, N-methyl-N,N-dioleylamine, and N,N-dimethyloleylamine.

         In another group of embodiments, the amino lipid is a lipid in which the amino head group is attached to one or more fatty acid or fatty alkyl groups by a scaffold such as, for example, a glycerol or propanediol moiety. Illustrative of these amine lipids is the formula:

25

$$R^{11}O\diagdown_{}\underset{R^{12}O\diagup}{}\diagup\!\!\diagdown\!\!-N\!\!\underset{R^{14}}{\overset{R^{13}}{\diagup}}$$

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wherein at least one and preferably both of R^{11} and R^{12} is a C_{12} to C_{24} alkyl or acyl group which is branched or unbranched, saturated or unsaturated. In those embodiments in which only one of R^{11} or R^{12} is a long chain alkyl or acyl group, the other of R^{11} or R^{12} will be a hydrogen or lower alkyl or acyl group having from one to six carbon atoms. The remaining

5   groups, R^{13} and R^{14} are typically hydrogen or C_1 to C_4 alkyl. In this group of embodiments, the amino lipid can be viewed as a derivative of 3-monoalkyl or dialkylamino-1,2-propanediol. An example of a suitable amino lipid is DODAP (1,2-dioleoyloxy-3-dimethylamino-propane, see Figure 2A). Other amino lipids would include those having alternative fatty acid groups and other dialkylamino groups, including

10   those in which the alkyl substitutents are different (*e.g.*, N-ethyl-N-methylamino-, N-propyl-N-ethylamino- and the like). For those embodiments in which R^{11} and R^{12} are both long chain alkyl or acyl groups, they can be the same or different. In general, amino lipids having less saturated acyl chains are more easily sized, particularly when the complexes must be sized below about 0.3 microns, for purposes of filter sterilization. Amino lipids containing

15   unsaturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are particularly preferred. Other scaffolds can also be used to separate the amino group and the fatty acid or fatty alkyl portion of the amino lipid. Suitable scaffolds are known to those of skill in the art.

       Compounds that are related to DODAP that may be useful with this invention include: 1-oleoyl-2-hydroxy-3-N,N-dimethylamino propane; 1,2-diacyl-3-N,N-

20   dimethylamino propane; and 1,2-didecanoyl-1-N,N-dimethylamino propane. Further, it is proposed that various modifications of the DODAP or DODMA headgroup, or any compound of the general formula:

can be modified to obtain a suitable pKa. Suitable headgroup modifications that are useful in the instant invention include:

25

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	R^1	R^2
1	H	H
2	H	CH_3
3	CH_3	CH_3
4	H	CH_2CH_3
5	CH_3	CH_2CH_3
6	CH_2CH_3	CH_2CH_3
7	H	CH_2CH_2OH
8	CH_3	CH_2CH_2OH
9	CH_2CH_3	CH_2CH_2OH
10	CH_2CH_2OH	CH_2CH_2OH
11*	H	$CH_2CH_2NH_2$
12*	CH_3	$CH_2CH_2NH_2$
13*	CH_2CH_3	$CH_2CH_2NH_2$
14*	CH_2CH_2OH	$CH_2CH_2NH_2$
15*	$CH_2CH_2NH_2$	$CH_2CH_2NH_2$

In other embodiments, the amino lipid can be a derivative of a naturally occurring amino lipid, for example, sphingosine. Suitable derivatives of sphingosine would include those having additional fatty acid chains attached to either of the pendent hydroxyl groups, as well as alkyl groups, preferably lower alkyl groups, attached to the amino functional group.

Other lipids which may be used as the first lipid component of the invention include phosphine lipids (although toxicity issues may limit their utility), and carboxylic acid lipid derivative. These generally have a pKa of about 5 and are therefore useful with cationic therapeutic agents.

The second lipid component is selected to improve the formulation process by reducing aggregation of the lipid particles during formation. This may result from steric stabilization of particles which prevents charge-induced aggregation during formation. Examples of suitable lipids for this purpose include polyethylene glycol (PEG)-modified lipids, monosialoganglioside Gm1, and polyamide oligomers ("PAO") such as ATTA

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(disclosed in US Pat. Appl. SN 60/073,852 and US Pat. Appl. SN 60/(not yet received TT&C

Attorney Docket No.16303-005810 both assigned to the assignee of the instant invention and

incorporated herein by reference). Other compounds with uncharged, hydrophilic, steric-

barrier moieties, that prevent aggregation during formulation, like PEG, Gm1 or ATTA, can

5   also be coupled to lipids for use as the second lipid component in the methods and

compositions of the invention. Typically, the concentration of the second lipid component is

about 1 to 15% (by mole percent of lipids).

        Specific examples of PEG-modified lipids (or lipid-polyoxyethylene

conjugates) that are useful in the present invention can have a variety of "anchoring" lipid

10   portions to secure the PEG portion to the surface of the lipid vesicle. Examples of suitable

PEG-modified lipids include PEG-modified phosphatidylethanolamine and phosphatidic acid

(see Figure 2B, structures A and B), PEG-modified diacylglycerols and dialkylglycerols (see

Figure 2B, structures C and D), PEG-modified dialkylamines (Figure 2B, structure E) and

PEG-modified 1,2-diacyloxypropan-3-amines (Figure 2B, structure F). Particularly preferred

15   are PEG-ceramide conjugates (*e.g.,* PEG-CerC14 or PEG-CerC20) which are described in co-

pending USSN 08/486,214, incorporated herein by reference.

        In embodiments where a sterically-large moiety such as PEG or ATTA are

conjugated to a lipid anchor, the selection of the lipid anchor depends on what type of

association the conjugate is to have with the lipid particle. It is well known that

20   mePEG(mw2000)-diastearoyl-phosphatidylethanolamine (PEG-DSPE) will remain

associated with a liposome until the particle is cleared from the circulation, possibly a matter

of days. Other conjugates, such as PEG-CerC20 have similar staying capacity. PEG-

CerC14, however, rapidly exchanges out of the formulation upon exposure to serum, with a

$T_{1/2}$ less than 60 mins. in some assays. As illustrated in US Pat. Application SN 08/486,214

25   at least three characteristics influence the rate of exchange: length of acyl chain, saturation of

acyl chain, and size of the steric-barrier head group. Compounds having suitable variations

of these features may be useful for the invention.

        In addition to the first and second lipid components, the lipid mixture may

contain additional lipid species. These additional lipids may be, for example, neutral lipids or

30   sterols.

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Neutral lipids, when present in the lipid mixture, can be any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, for example diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, and cerebrosides. The

5   selection of neutral lipids for use in the complexes herein is generally guided by consideration of, *e.g.*, liposome size and stability of the liposomes in the bloodstream. Preferably, the neutral lipid component is a lipid having two acyl groups, (*i.e.*, diacylphosphatidylcholine and diacylphosphatidylethanolamine). Lipids having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be

10   isolated or synthesized by well-known techniques. In one group of embodiments, lipids containing saturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are preferred. In another group of embodiments, lipids with mono or diunsaturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are used. Additionally, lipids having mixtures of saturated and unsaturated fatty acid chains can be used. Preferably, the neutral

15   lipids used in the present invention are DOPE, DSPC, POPC, or any related phosphatidylcholine. The neutral lipids useful in the present invention may also be composed of sphingomyelin or phospholipids with other head groups, such as serine and inositol.

The sterol component of the lipid mixture, when present, can be any of those sterols conventionally used in the field of liposome, lipid vesicle or lipid particle preparation.

20   A preferred sterol is cholesterol.

The mixture of lipids is typically a solution of lipids in an alcoholic solvent. Hydrophilic, low molecular weight water miscible alcohols with less than 10 carbon atoms, preferably less than 6 carbon atoms are preferred. Typical alcohols used in this invention are ethanol, methanol, propanol, butanol, pentanol and ethylene glycol and propylene glycol.

25   Particularly preferred is ethanol. In most embodiments, the alcohol is used in the form in which it is commercially available. For example, ethanol can be used as absolute ethanol (100%), or as 95% ethanol, the remainder being water.

In one exemplary embodiment, the mixture of lipids is a mixture of amino lipids, neutral lipids (other than an amino lipid), a sterol (*e.g.*, cholesterol) and a PEG-

30   modified lipid (*e.g.*, a PEG-ceramide) in an alcohol solvent. In preferred embodiments, the lipid mixture consists essentially of an amino lipid, a neutral lipid, cholesterol and a PEG-

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ceramide in alcohol, more preferably ethanol. In further preferred embodiments, the first

solution consists of the above lipid mixture in molar ratios of about 10-35% amino lipid:25-

45% neutral lipid:35-55% cholesterol:0.5-15% PEG-ceramide. In still further preferred

embodiments, the first solution consists essentially of DODAP, DSPC, Chol and PEG-

5   CerC14, more preferably in a molar ratio of about 10-35% DODAP:25-45% DSPC:35-55%

Chol:0.5-15% PEG-CerC14. In another group of preferred embodiments, the neutral lipid in

these compositions is replaced with POPC or SM.

　　　　　In accordance with the invention, the lipid mixture is combined with a

buffered aqueous solution of charged therapeutic agent, preferably nucleic acids. The

10   buffered aqueous solution of therapeutic agents (nucleic acids) which is combined with the

lipid mixture is typically a solution in which the buffer has a pH of less than the pK_a of the

protonatable lipid in the lipid mixture. As used herein, the term "nucleic acid" is meant to

include any oligonucleotide or polynucleotide having from 10 to 100,000 nucleotide residues.

Antisense and ribozyme oligonucleotides are particularly preferred. The term "antisense

15   oligonucleotide" or simply "antisense" is meant to include oligonucleotides which are

complementary to a targeted nucleic acid and which contain from about 10 to about 50

nucleotides, more preferably about 15 to about 30 nucleotides. The term also encompasses

antisense sequences which may not be exactly complementary to the desired target gene.

Thus, the invention can be utilized in instances where non-target specific-activities are found

20   with antisense, or where an antisense sequence containing one or more mismatches with the

target sequence is the most preferred for a particular use.

　　　　　The nucleic acid that is used in a lipid-nucleic acid particle according to this

invention includes any form of nucleic acid that is known. Thus, the nucleic acid may be a

modified nucleic acid of the type used previously to enhance nuclease resistance and serum

25   stability. Surprisingly, however, acceptable therapeutic products can also be prepared using

the method of the invention to formulate lipid-nucleic acid particles from nucleic acids which

have no modification to the phosphodiester linkages of natural nucleic acid polymers, and the

use of unmodified phosphodiester nucleic acids (i.e., nucleic acids in which all of the linkages

are phosphodiester linkages) is a preferred embodiment of the invention. Still other nucleic

30   acids which are useful in the present invention include, synthetic or pre-formed poly-RNA

such as poly(IC) IC.

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The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or DNA-RNA hybrids. Examples of double-stranded DNA include structural genes, genes including control and termination regions, and self-replicating systems such as plasmid DNA. Single-stranded nucleic acids include antisense oligonucleotides (discussed

5   above and complementary to DNA and RNA), ribozymes and triplex-forming oligonucleotides.

In order to increase stability, some single-stranded nucleic acids may have some or all of the nucleotide linkages substituted with stable, non-phosphodiester linkages, including, for example, phosphorothioate, phosphorodithioate, phosphoroselenate,

10   boranophosphate, methylphosphonate, or O-alkyl phosphotriester linkages. Phosphorothioate nucleic acids (PS-oligos) are those oligonucleotides or polynucleotides in which one of the non-bridged oxygens of the internucleotide linkage has been replaced with sulfur. These PS-oligos are resistant to nuclease degradation, yet retain sequence-specific activity. Similarly, phosphorodithioate nucleic acids are those oligonucleotides or

15   polynucleotides in which each of the non-bridged oxygens of the internucleotide linkage have been replaced by a sulfur atom. These phosphorodithioate-oligos have also proven to be more nuclease resistant than the natural phosphodiester-linked form. Other useful nucleic acids derivatives include those nucleic acids molecules in which the bridging oxygen atoms (those forming the phosphoester linkages) have been replaced with $-S-$, $-NH-$, $-CH_2-$ and the

20   like. Preferably, the alterations to the antisense or other nucleic acids used will not completely affect the negative charges associated with the nucleic acids. Thus, the present invention contemplates the use of antisense and other nucleic acids in which a portion of the linkages are replaced with, for example, the neutral methyl phosphonate or phosphoramidate linkages. When neutral linkages are used, preferably less than 80% of the nucleic acid

25   linkages are so substituted, more preferably less than 50%.

Those skilled in the art will realize that for *in vivo* utility, such as therapeutic efficacy, a reasonable rule of thumb is that if a thioated version of the sequence works in the free form, that encapsulated particles of the same sequence, of any chemistry, will also be efficacious. Encapsulated particles may also have a broader range of *in vivo* utilities,

30   showing efficacy in conditions and models not known to be otherwise responsive to antisense therapy. Those skilled in the art know that applying this invention they may find old models

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which now respond to antisense therapy. Further, they may revisit discarded antisense sequences or chemistries and find efficacy by employing the invention.

Therapeutic antisense sequences (putatatively target specific) known to work with this invention include the following:

5

Trivial Name: Gene Target, Chemistry and Sequence

PS-3082   murine ICAM-1 (Intracellular Adhesion Molecule-1) (phosphorothioate)

10   TGCATCCCCCAGGCCACCAT   (SEQ ID. No 1)

PO-3082   murine ICAM-1 (phosphodiester)

                 TGCATCCCCCAGGCCACCAT   (SEQ ID. No 1)

PS-2302   human ICAM-1 (phosphorothioate)

                 GCCCAAGCTGGCATCCGTCA   (SEQ ID. No 2)

15   PO-2302   human ICAM-1 (phosphodiester)

                 GCCCAAGCTGGCATCCGTCA   (SEQ ID. No 2)

PS-8997   human ICAM-1 (phosphorothioate)

                 GCCCAAGCTGGCATCCGTCA   (SEQ ID. No 2)

PO- 8997   human ICAM-1 (phosphodiester)

20   GCCCAAGCTGGCATCCGTCA   (SEQ ID. No 2)

US3   human erb-B-2 gene (phosphodiester or phosphorothioate)

                 GGT GCT CAC TGC GGC   (SEQ ID. No 3)

LR-3280   human c-myc gene (phosphorothioate)

                 AAC GTT GAG GGG CAT   (SEQ ID. No 4)

25   Inx-6298   human c-myc gene (phosphodiester)

                 AAC GTT GAG GGG CAT   (SEQ ID. No 4)

Inx-6295   human c-myc gene (phosphodiester or phosphorothioate)

                 T AAC GTT GAG GGG CAT   (SEQ ID. No 5)

LR-3001   human c-myb gene (phosphodiester or phosphorothioate)

30   TAT GCT GTG CCG GGG TCT TCG GGC   (SEQ ID. No 6)

c-myb   human c-myb gene (phosphodiester or phosphorothioate)

                 GTG CCG GGG TCT TCG GGC   (SEQ ID. No 7)

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IGF-1R   human IGF-1R (Insulin Growth Factor 1 – Receptor)

(phosphodiester or phosphorothioate)

GGA CCC TCC TCC GGA GCC   (SEQ ID. No 8)

LR-42   human IGF-1R   (phosphodiester or phosphorothioate)

5   TCC TCC GGA GCC AGA CTT   (SEQ ID. No 9)

EGFR   human EGFR (Epidermal Growth Factor Receptor)
(phosphodiester or phosphorothioate)

CCG TGG TCA TGC TCC   (SEQ ID. No 10)

VEGF   human VEGF (Vascular Endothelial Growth Factor) gene
10   (phosphodiester or phosphorothioate)

CAG CCT GGC TCA CCG CCT TGG   (SEQ ID. No 11)

PS-4189   murine PKC-alpha (Phosphokinase C – alpha) gene
(phosphodiester or phosphorothioate)

CAG CCA TGG TTC CCC CCA AC   (SEQ ID. No 12)

15   PS-3521   human PKC-alpha
(phosphodiester or phosphorothioate)

GTT CTC GCT GGT GAG TTT CA   (SEQ ID. No 13)

Bcl-2   human bcl-2 gene
(phosphodiester or phosphorothioate)

20   TCT CCC AgC gTg CgC CAT   (SEQ ID. No 14)

ATG-AS   human c-*raf*-1 protein kinase
(phosphodiester or phosphorothioate)

GTG CTC CAT TGA TGC   (SEQ ID. No 15)

VEGF-R1   human VEGF-R –1 (Vascular Endothelial Growth Factor Receptor 1)
25   ribozyme

GAG UUG CUG AUG AGG CCG AAA GGC CGA AAG UCU G

(SEQ ID. No 16)

Using these sequences, the invention provides a method for the treatment of a

diseases, including tumors, characterized by aberrant expression of a gene in a mammalian

30   subject. The method comprises the steps of preparing a lipid-encapsulated therapeutic

nucleic acid particle according to the methods as described herein, where the therapeutic

nucleic acid component hybridizes specifically with the aberrantly expressed gene; and

administering a therapeutically effective amount of the resulting particle to the mammalian

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subject. These sequences are, of course, only representative of the possible therapeutic oligonucleotide compounds that can be delivered using the invention. It is well known that, depending on the target gene, antisense that hybridizes to any part of the target gene, such as coding regions, introns, the 5' untranslated region (5'UTR), start of translation, or 3'UTR

5   may have therapeutic utility. Therefore, the sequences listed above are only exemplary of antisense. Furthermore, all the alternative chemistries that have been proposed (i.e. see Background) can be tested with the invention to determine efficacy along with all types of ribozymes. In short, the compounds listed above represent the broad class of therapeutic 5-50 mer oligonucleotides of various chemistries which are useful with this invention. Other

10   oligonucleotides which are useful include all those which have previously demonstrated efficacy in the free form.

While the invention is generally described and exemplified with regard to antisense oligonucleotides, other nucleic acids can be formulated and administered to a subject for the purpose of repairing or enhancing the expression of a cellular protein.

15   Accordingly, the nucleic acid can be an expression vector, cloning vector or the like which is often a plasmid designed to be able to replicate in a chosen host cell. Expression vectors may replicate autonomously, or they may replicate by being inserted into the genome of the host cell, by methods well known in the art. Vectors that replicate autonomously will have an origin of replication or autonomous replicating sequence (ARS) that is functional in the

20   chosen host cell(s). Often, it is desirable for a vector to be usable in more than one host cell, e.g., in *E. coli* for cloning and construction, and in a mammalian cell for expression.

Additionally, the nucleic acid can carry a label (*e.g.,* radioactive label, fluorescent label or colorimetric label) for the purpose of providing clinical diagnosis relating to the presence or absence of complementary nucleic acids. Accordingly, the nucleic acids,

25   or nucleotide polymers, can be polymers of nucleic acids including genomic DNA, cDNA, mRNA or oligonucleotides containing nucleic acid analogs, for example, the antisense derivatives described in a review by Stein, *et al., Science* **261**:1004-1011 (1993) and in U.S. Patent Nos. 5,264,423 and 5,276,019, the disclosures of which are incorporated herein by reference. Still further, the nucleic acids may encode transcriptional and translational

30   regulatory sequences including promoter sequences and enhancer sequences.

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The nucleic acids used in the present invention will also include those nucleic acids in which modifications have been made in one or more sugar moieties and/or in one or more of the pyrimidine or purine bases. Examples of sugar modifications include replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, azido

5   groups or functionalized as ethers or esters. Additionally, the entire sugar may be replaced with sterically and electronically similar structures, including aza-sugars and carbocyclic sugar analogs. Modifications in the purine or pyrimidine base moiety include, for example, alkylated purines and pyrimidines, acylated purines or pyrimidines, or other heterocyclic substitutes known to those of skill in the art. As with the modifications to the phosphodiester

10   linkages discussed above, any modifications to the sugar or the base moieties should also act to preserve at least a portion of the negative charge normally associated with the nucleic acid. In particular, modifications will preferably result in retention of at least 10% of the overall negative charge, more preferably over 50% of the negative charge and still more preferably over 80% of the negative charge associated with the nucleic acid.

15   The nucleic acids used in the present method can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries or prepared by synthetic methods. Synthetic nucleic acids can be prepared by a variety of solution or solid phase methods. Generally, solid phase synthesis is preferred. Detailed descriptions of the procedures for solid phase synthesis of nucleic acids by phosphite-triester, phosphotriester,

20   and H-phosphonate chemistries are widely available. See, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers, *et al.,* U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage, *et al., Tetrahedron Lett.,* **22**:1859-1862 (1981); Matteucci, *et al., J. Am. Chem. Soc.,* **103**:3185-3191 (1981); Caruthers, *et al., Genetic Engineering,* **4**:1-17 (1982); Jones, chapter 2, Atkinson, *et al.,* chapter 3, and Sproat, *et al.,* chapter 4, in *Oligonucleotide Synthesis: A Practical*

25   *Approach,* Gait (ed.), IRL Press, Washington D.C. (1984); Froehler, *et al., Tetrahedron Lett.,* **27**:469-472 (1986); Froehler, *et al., Nucleic Acids Res.,* **14**:5399-5407 (1986); Sinha, *et al. Tetrahedron Lett.,* **24**:5843-5846 (1983); and Sinha, *et al., Nucl. Acids Res.,* **12**:4539-4557 (1984) which are incorporated herein by reference.

As noted above, the solution of therapeutic agent (nucleic acids) comprises an

30   aqueous buffer. Preferred buffers (in the case of anionic therapeutic agents) are those which provide a pH of less than the pK_a of the first lipid component. Examples of suitable buffers

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include citrate, phosphate, acetate, and MES. A particularly preferred buffer is citrate buffer.

Preferred buffers will be in the range of 1-1000 mM of the anion, depending on the chemistry of the oligonucleotide being encapsulated, and optimization of buffer concentration may be significant to achieving high loading levels (See. Figs 15 and 20).   Alternatively,

5   pure water acidified to pH 5-6 with chloride, sulfate or the like may be useful. In this case, it may be suitable to add 5% glucose, or another non-ionic solute which will balance the osmotic potential across the particle membrane when the particles are dialyzed to remove ethanol, increase the pH, or mixed with a pharmaceutically acceptable carrier such as normal saline. The amount of therapeutic agent (nucleic acid) in buffer can vary, but will typically

10   be from about 0.01 mg/mL to about 200 mg/mL, more preferably from about 0.5 mg/mL to about 50 mg/mL.

The mixture of lipids and the buffered aqueous solution of therapeutic agent (nucleic acids) is combined to provide an intermediate mixture. The intermediate mixture is typically a mixture of lipid particles having encapsulated therapeutic agent (nucleic acids).

15   Additionally, the intermediate mixture may also contain some portion of therapeutic agent (nucleic acids) which are attached to the surface of the lipid particles (liposomes or lipid vesicles) due to the ionic attraction of the negatively-charged nucleic acids and positively-charged lipids on the lipid particle surface (the amino lipids or other lipid making up the protonatable first lipid component are positively charged in a buffer having a pH of less than

20   the pK_a of the protonatable group on the lipid). In one group of preferred embodiments, the mixture of lipids is an alcohol solution of lipids and the volumes of each of the solutions is adjusted so that upon combination, the resulting alcohol content is from about 20% by volume to about 45% by volume. The method of combining the mixtures can include any of a variety of processes, often depending upon the scale of formulation produced. For example,

25   when the total volume is about 10-20 mL or less, the solutions can be combined in a test tube and stirred together using a vortex mixer. Large-scale processes can be carried out in suitable production scale glassware.

Optionally, the lipid-encapsulated therapeutic agent (nucleic acid) complexes which are produced by combining the lipid mixture and the buffered aqueous solution of

30   therapeutic agents (nucleic acids) can be sized to achieve a desired size range and relatively narrow distribution of lipid particle sizes. Preferably, the compositions provided herein will

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be sized to a mean diameter of from about 70 to about 200 nm, more preferably about 90 to about 130 nm. Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive

5   size reduction down to small unilamellar vesicles (SUVs) less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size

10   distribution can be monitored by conventional laser-beam particle size determination. For the methods herein, extrusion is used to obtain a uniform vesicle size.

Extrusion of liposome compositions through a small-pore polycarbonate membrane or an asymmetric ceramic membrane results in a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times

15   until the desired liposome complex size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. In some instances, the lipid-nucleic acid compositions which are formed can be used without any sizing.

The present invention further comprises a step of neutralizing at least some of

20   the surface charges on the lipid portions of the lipid-nucleic acid compositions. By at least partially neutralizing the surface charges, unencapsulated antisense or other nucleic acid is freed from the lipid particle surface and can be removed from the composition using conventional techniques. Preferably, unencapsulated and surface adsorbed nucleic acids is removed from the resulting compositions through exchange of buffer solutions. For example,

25   replacement of a citrate buffer (pH about 4.0, used for forming the compositions) with a HEPES-buffered saline (HBS pH about 7.5) solution, results in the neutralization of liposome surface and antisense release from the surface. The released antisense can then be removed via chromatography using standard methods, and then switched into a buffer with a pH above the pKa of the lipid used.

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In other aspects, the present invention provides lipid-encapsulated nucleic acid compositions, preferably prepared by the methods recited above. Accordingly, preferred compositions are those having the lipid ratios and nucleic acid preferences noted above.

In still other aspects, the present invention contemplates reversed-charge methods in which the lipid portion of the complex contains certain anionic lipids and the component which is encapsulated is a positively charged therapeutic agent. One example of a positively charged agent is a positively charged peptide or protein. In essentially an identical manner, liposome-encapsulated protein is formed at a pH above the pKa of the anionic lipid, then the surface is neutralized by exchanging the buffer with a buffer of lower pH (which would also release surface-bound peptide or protein).

IV.   **Pharmaceutical Preparations**

The lipid-nucleic acid compositions prepared by the above methods can be administered either alone or in mixture with a physiologically-acceptable carrier (such as physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice.

Pharmaceutical compositions comprising the lipid-nucleic acid compositions of the invention are prepared according to standard techniques and further comprise a pharmaceutically acceptable carrier. Generally, normal saline will be employed as the pharmaceutically acceptable carrier. Other suitable carriers include, e.g., water, buffered water, 0.9% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* In compositions comprising saline or other salt containing carriers, the carrier is preferably added following lipid particle formation. Thus, after the lipid-nucleic acid compositions are formed, the compositions can be diluted into pharmaceutically acceptable carriers such as normal saline. The resulting pharmaceutical preparations may be sterilized by conventional, well known sterilization techniques. The aqueous solutions can then be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium

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lactate, sodium chloride, potassium chloride, calcium chloride, *etc.* Additionally, the lipidic suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as α-tocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

5   The concentration of lipid-nucleic acid complexes in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.01%, usually at or at least about 0.05-5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with

10   treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, complexes composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration. In one group of embodiments, the nucleic acid will have an attached label and will be used for diagnosis (by indicating the presence of complementary nucleic acid). In

15   this instance, the amount of complexes administered will depend upon the particular label used, the disease state being diagnosed and the judgement of the clinician but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight.

          As noted above, the lipid-therapeutic agent (nucleic acid) compositions of the

20   invention include polyethylene glycol (PEG)-modified phospholipids, PEG-ceramide, or ganglioside G_{M1}-modified lipids or other lipids effective to prevent or limit aggregation. Addition of such components does not merely prevent complex aggregation, however, it may also provides a means for increasing circulation lifetime and increasing the delivery of the lipid-nucleic acid composition to the target tissues.

25   The present invention also provides lipid-nucleic acid compositions in kit form. The kit will typically be comprised of a container which is compartmentalized for holding the various elements of the kit. The kit will contain the compositions of the present inventions, preferably in dehydrated or concentrated form, with instructions for their rehydration or dilution and administration. In still other embodiments, the lipid-

30   encapsulated-therapeutic agent (nucleic acid) particles will have a targeting moiety attached to the surface of the lipid particle. Methods of attaching targeting moieties (*e.g.*, antibodies,

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proteins, small molecule mimetics, vitamins, oligosaccharides and hyaluronic acid) to lipids (such as those used in the present compositions) are known to those of skill in the art.

Dosage for the lipid-nucleic acid compositions will depend on the ratio of nucleic acid to lipid and the administrating physician's opinion based on age, weight, and

5   condition of the patient.

V.   Methods of Introducing Lipid-Encapsulated Therapeutic Agents Into Cells

The lipid-therapeutic agent compositions of the invention can be used for introduction of those therapeutic agents into cells. In the case of nucleic acid-containing

10   compositions, the composition of the invention are useful for the introduction of nucleic acids, preferably plasmids, antisense and ribozymes into cells. Accordingly, the present invention also provides methods for introducing a therapeutic agent such as a nucleic acid into a cell. The methods are carried out *in vitro* or *in vivo* by first forming the compositions as described above, then contacting the compositions with the target cells for a period of time

15   sufficient for transfection to occur.

The compositions of the present invention can be adsorbed to almost any cell type. Once adsorbed, the complexes can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the nucleic acid portion of the complex can take place via any one of these pathways. In

20   particular, when fusion takes place, the liposome membrane is integrated into the cell membrane and the contents of the liposome combine with the intracellular fluid. Contact between the cells and the lipid-nucleic acid compositions, when carried out *in vitro*, will take place in a biologically compatible medium. The concentration of compositions can vary widely depending on the particular application, but is generally between about 1 μmol and

25   about 10 mmol. Treatment of the cells with the lipid-nucleic acid compositions will generally be carried out at physiological temperatures (about 37°C) for periods of time of from about 1 to 6 hours, preferably of from about 2 to 4 hours. For *in vitro* applications, the delivery of nucleic acids can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the

30   cells will be animal cells, more preferably mammalian cells, and most preferably human cells.

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In one group of preferred embodiments, a lipid-nucleic acid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/mL, more preferably about 2×10^4 cells/mL. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 µg/mL, more preferably

5   about 0.1 µg/mL.

Typical applications include using well known transfection procedures to provide intracellular delivery of DNA or mRNA sequences which code for therapeutically useful polypeptides. In this manner, therapy is provided for genetic diseases by supplying deficient or absent gene products (*i.e.*, for Duchenne's dystrophy, see Kunkel, *et al., Brit.*

10   *Med. Bull.* **45(3)**:630-643 (1989), and for cystic fibrosis, see Goodfellow, *Nature* **341**:102-103 (1989)). Other uses for the compositions of the present invention include introduction of antisense oligonucleotides in cells (see, Bennett, *et al., Mol. Pharm.* **41**:1023-1033 (1992)).

Alternatively, the compositions of the present invention can also be used for the transfection of cells *in vivo*, using methods which are known to those of skill in the art. In

15   particular, Zhu, *et al., Science* **261**:209-211 (1993), incorporated herein by reference, describes the intravenous delivery of cytomegalovirus (CMV)-chloramphenicol acetyltransferase (CAT) expression plasmid using DOTMA-DOPE complexes. Hyde, *et al., Nature* **362**:250-256 (1993), incorporated herein by reference, describes the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to epithelia of the airway

20   and to alveoli in the lung of mice, using liposomes. Brigham, *et al., Am. J. Med. Sci.* **298**:278-281 (1989), incorporated herein by reference, describes the *in vivo* transfection of lungs of mice with a functioning prokaryotic gene encoding the intracellular enzyme, chloramphenicol acetyltransferase (CAT). Thus, the compositions of the invention can be used in the treatment of infectious diseases.

25   For *in vivo* administration, the pharmaceutical compositions are preferably administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. More preferably, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection. For example, see Stadler, *et al.*, U.S. Patent No. 5,286,634, which is incorporated herein by reference.

30   Intracellular nucleic acid delivery has also been discussed in Straubringer, *et al.*, METHODS IN ENZYMOLOGY, Academic Press, New York. **101**:512-527 (1983); Mannino, *et al.*,

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Biotechniques **6**:682-690 (1988); Nicolau, *et al., Crit. Rev. Ther. Drug Carrier Syst.*
6:239-271 (1989). and Behr, *Acc. Chem. Res.* **26**:274-278 (1993). Still other methods of
administering lipid-based therapeutics are described in, for example, Rahman *et al.,* U.S.
Patent No. 3,993,754; Sears, U.S. Patent No. 4,145,410; Papahadjopoulos *et al.,* U.S. Patent

5   No. 4,235,871; Schneider, U.S. Patent No. 4,224,179; Lenk *et al.,* U.S. Patent No. 4,522,803;
and Fountain *et al.,* U.S. Patent No. 4,588,578.

In other methods, the pharmaceutical preparations may be contacted with the
target tissue by direct application of the preparation to the tissue. The application may be
made by topical, "open" or "closed" procedures. By "topical", it is meant the direct

10   application of the pharmaceutical preparation to a tissue exposed to the environment, such as
the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those
procedures which include incising the skin of a patient and directly visualizing the underlying
tissue to which the pharmaceutical preparations are applied. This is generally accomplished
by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to

15   access abdominal viscera, or other direct surgical approach to the target tissue. "Closed"
procedures are invasive procedures in which the internal target tissues are not directly
visualized, but accessed via inserting instruments through small wounds in the skin. For
example, the preparations may be administered to the peritoneum by needle lavage.
Likewise, the pharmaceutical preparations may be administered to the meninges or spinal

20   cord by infusion during a lumbar puncture followed by appropriate positioning of the patient
as commonly practiced for spinal anesthesia or metrazamide imaging of the spinal cord.
Alternatively, the preparations may be administered through endoscopic devices.

The lipid-nucleic acid compositions can also be administered in an aerosol
inhaled into the lungs (see, Brigham, *et al., Am. J. Sci.* **298(4)**:278-281 (1989)) or by direct

25   injection at the site of disease (Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc.,
Publishers, New York. pp.70-71 (1994)).

The methods of the present invention may be practiced in a variety of hosts.
Preferred hosts include mammalian species, such as humans, non-human primates, dogs, cats,
cattle, horses, sheep, and the like.

30

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VI.   Examples

Materials and Methods:

Lipids

Distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), and

5   palmitoyloleoylphosphatidylcholine (POPC) were purchased from Northern Lipids

(Vancouver, Canada). 1,2-dioleoyloxy-3-dimethylammoniumpropane (DODAP or AL-1)

was synthesized by Dr. Steven Ansell (Inex Pharmaceuticals) or, alternatively, was purchased

from Avanti Polar Lipids. Cholesterol was purchased from Sigma Chemical Company (St.

Louis, Missouri, USA). PEG-ceramides were synthesized by Dr. Zhao Wang at Inex

10   Pharmaceuticals Corp. using procedures described in PCT WO 96/40964, incorporated herein

by reference. [^{3}H] or [^{14}C]-CHE was purchased from NEN (Boston, Massachusetts, USA).

All lipids were > 99% pure.

Buffers and Solvents

15   Ethanol (95%), methanol, chloroform, citric acid, HEPES and NaCl were all

purchased from commercial suppliers.

Synthesis and Purification of Phosphorothioate Antisense

PS 3082, a 20mer phosphorothioate antisense oligodeoxynucleotide, was

20   synthesized, purified and donated by ISIS Pharmaceuticals (Carlsbad, California, USA). The

sequence for this oligo is: TGCATCCCCCAGGCCACCAT. (Seq ID No 1) The details of

the synthesis and purification can be found elsewhere (see, Stepkowski, *et al., J. Immunol.*

153:5336-5346 (1994)).

Preparation of Liposomal Antisense

25   Lipid stock solutions were prepared in 95% ethanol at 20 mg/mL

(PEG-Ceramides were prepared at 50 mg/mL). DSPC, CHOL, DODAP, PEG-CerC14

(25:45:20:10, molar ratio), 13 μmol total lipid, were added to a 13 x 100 mm test tube

containing trace amounts of [^{14}C]-cholesterylhexadecylether. The final volume of the lipid

30   mixture was 0.4 mL. In some experiments, SM or POPC was substituted for DSPC. A

20mer antisense oligodeoxynucleotide, PS 3082 (2 mg), and trace amounts of [^{3}H]-PS 3082

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were dissolved in 0.6 mL of 300 mM citric acid, pH 3.8 in a separate 13 x 100 mm test tube. The antisense solution was warmed to 65°C and the lipids (in ethanol) were slowly added, mixing constantly. The resulting volume of the mixture was 1.0 mL and contained 13 µmol total lipid, 2 mg of antisense oligodeoxynucleotide, and 38% ethanol, vol/vol. The

5   antisense-lipid mixture was subjected to 5 cycles of freezing (liquid nitrogen) and thawing (65°C), and subsequently was passed 10X through three stacked 100 nm filters (Poretics) using a pressurized extruder apparatus with a thermobarrel attachment (Lipex Biomembranes). The temperature and pressure during extrusion were 65°C and 300-400 psi (nitrogen), respectively. The extruded preparation was diluted with 1.0 mL of 300 mM citric

10   acid, pH 3.8, reducing the ethanol content to 20%. The preparation was immediately applied to a gel filtration column. Alternatively, the extruded sample was dialyzed (12 000-14 000 MW cutoff; SpectraPor) against several liters of 300 mM citrate buffer, pH 3.8 for 3-4 hours to remove the excess ethanol. The sample was subsequently dialyzed against HBS, pH 7.5, for 12-18 hours to neutralize the DODAP and release any antisense that was associated with

15   the surface of the vesicles. The free antisense was removed from the encapsulated liposomal antisense by gel exclusion chromatography as described below.

Gel Filtration Chromatography

A 20 x 2.5 cm glass column containing Biogel A15m, 100-200 mesh, was

20   equilibrated in HEPES-buffered saline (HBS; 20 mM HEPES, 145 mM NaCl, pH 7.5). The 2.0 mL liposomal antisense preparation was applied to the column and allowed to drain into the gel bed under gravity. The column was eluted with HBS at a flow rate of 50 mL/hr. Column fractions (1.0 mL) were collected and analyzed for radioactivity using standard liquid scintillation counting techniques. The fractions were pooled based on the levels of

25   [^{14}C]-CHE present in the fraction. The size distribution of the pooled liposomal antisense was determined using a NICOMP Model 370 Sub-micron particle sizer and was typically 110 ± 30 nm.

Ion Exchange Chromatography

30   As an alternative to gel filtration chromatography, samples were sometimes dialyzed first in 300 mM citrate, pH 3.80, for 2-3 hours to remove residual ethanol, followed

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by at least a 12 hour dialysis in HBS, to exchange the external citrate for HBS and remove residual ethanol. The sample was applied to a 1.5 x 8 cm DEAE-Sepharose® column equilibrated in HBS. Free oligonucleotide binds to the DEAE with very high affinity. The peak containing the lipid was pooled, concentrated, and analyzed for antisense content, as
5   described below.

Assessment of Antisense Encapsulation

Antisense encapsulation was typically assessed by dual label ([^{3}H]-antisense and [^{14}C]-lipid) liquid scintillation counting after gel filtration chromatography to separate the
10   free and encapsulated antisense. Antisense encapsulation was evaluated by summing the total [^{3}H]-antisense radioactivity associated with the lipid peak and dividing by the total [^{3}H]-antisense radioactivity. Alternatively, the [^{3}H]/[^{14}C] ratio was determined before and after (*i.e.*, in the pooled lipid peak) gel filtration chromatography. Antisense encapsulation was also assessed by measuring the absorbance of the sample at 260 nm, preceded by a Bligh
15   and Dyer extraction of the antisense from the lipid, as described below.

Extraction of the Antisense

The antisense was extracted from the lipid according to the procedure outlined by Bligh and Dyer (Bligh, *et al., Can. J. Biochem. Physiol.* **37**:911-917 (1959)). Briefly, up
20   to 250 µL of aqueous sample was added to a 13 x 100 mm glass test tube, followed by the addition of 750 µL of chloroform:methanol (1:2.1, vol/vol), 250 µL of chloroform, and 250 µL of distilled water. The sample was mixed after each addition. The sample was centrifuged for 10 min. at 3000 rpm, resulting in a clear two-phase separation. The aqueous phase (top) was removed into a new 13 x 100 mm test tube. An aliquot (500 µL) of this
25   phase was diluted with 500 µL of distilled water, mixed, and the absorbance at 260 nm was assessed using a spectrophotometer. In some instances, the organic phase (bottom) was washed with 250 µL of methanol, centrifuged for 10 min. at 3000 rpm, and the upper phase removed and discarded. This was repeated 3 times. The washed organic phase was assessed for phospholipid content according to the method of Fiske and Subbarrow (Fiske, *et al., J.*
30   *Biol. Chem.* **66**:375-400 (1925)).

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OLIGREEN Assay

A fluorescent dye binding assay for quantifying single stranded oligonucleotide in aqueous solutions was established using a Biolumin™ 960 fluorescent plate reader (Molecular Dynamics, Sunnyvale, California, USA). Briefly, aliquots of encapsulated oligonucleotide were diluted in HEPES buffered saline (HBS; 20mM HEPES, 145mM NaCl, pH 7.5) . A 10 µL aliquot of the diluted sample was added to 100 µL of a 1:200 dilution of Oligreen™ reagent, both with and without 0.1% of Triton X-100 detergent. An oligo standard curve was prepared with and without 0.1% Triton X-100 for quantification of encapsulated oligo. Fluorescence of the OLIGREEN™-antisense complex was measured using excitation and emission wavelengths of 485nm and 520nm, respectively. Surface associated antisense was determined by comparing the fluorescence measurements in the absence and presence of detergent.

Ear Inflammation Model and Efficacy Studies

Sensitization and Elicitation of Contact Sensitivity

Mice were sensitized by applying 25 µL of 0.5% 2,4-dinitro-1-fluorobenzene (DNFB) in acetone:olive oil (4:1) to the shaved abdominal wall for two consecutive days. Four days after the second application, mice were challenged on the dorsal surface of the left ear with 10 µL of 0.2% DNFB in acetone:olive oil (4:1). Mice received no treatment on the contralateral (right) ear. In some cases, control mice received 10 µL of vehicle on the dorsal surface of the left ear.

Evaluation of Ear Swelling

Ear thickness was measured immediately prior to ear challenge, and at various time intervals after DNFB challenge, using an engineer's micrometer (Mitutoyo, Tokyo, Japan). Increases in ear thickness measurements were determined by subtracting the pre-challenge from post-challenge measurements.

The progression of ear inflammation over a 3 day period for ICR (outbred) mice is indicated in Figures 12 and 13. Erythema was evident almost immediately after ear challenge and gradually declined in intensity over the remainder of the study. ICR mice exhibited peak ear thickness at 24 hours after the induction of ear inflammation. Maximal ear thickness measurements were found to be 170×10^{-4} inches, corresponding to a 70% increase

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in ear thickness. Although ear swelling gradually declines at 48 and 72 hours after inflammation initiation, ear measurements still have not returned to baseline thickness levels (90-100 x 10^{-4} inches).

  The mouse *in vivo* experimental systems in this specification were selected in part because of their high degree of correlation to human disease conditions. The mouse ear inflammation model , which can be treated using methods and compositions of the invention, is well known to be an excellent model for human allergic contact dermatitis and other disease conditions. The control therapeutic used in this model is a corticosteroid which demonstrates efficacy both in the mouse model and in related human disease conditions.

  The mouse B16 tumor model, a fast growing melanoma, which can be treated using methods and compositions of the invention, is a standard, widely used experimental system. This tumor model can be successfully treated using vinca alkaloids, such as vincristine or vinblastine, which are known to be efficacious against human tumors as well.

  Treatments which demonstrate utility in the mouse models of this invention are excellent candidates for testing against human disease conditions, at similar dosages and administration modalities.

EXAMPLE 1

  This example illustrates the effects of ethanol on the encapsulation of antisense.

  A 20mer of [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The final ethanol concentration in the preparations was varied between 0 and 60%, vol/vol. The samples were extruded ten times through three 100 nm filters as described in "Materials and Methods". The samples were dialyzed for 2-3 hours in 300 mM citrate buffer, pH 3.80, to remove a majority of the excess ethanol. The samples were switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. This renders the majority of DODAP in the outer bilayer neutral, and will release any surface bound antisense. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose

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chromatography as described in "Material and Methods". Encapsulation was assessed either by analyzing the pre-column and post-column ratios of $[^3H]$-antisense and $[^{14}C]$-lipid or by determining the total pre-column and post-column $[^3H]$-antisense and $[^{14}C]$-lipid radioactivity.

5   In another experiment, the formulations were prepared as described. After extrusion, the filters were analyzed for $[^3H]$-antisense and $[^{14}C]$-lipid radioactivity by standard scintillation counting techniques. Results were expressed as a percent of the total initial radioactivity.

          Figure 3 demonstrates the effects of ethanol on the encapsulation of antisense 10   at pH 3.8. The encapsulation efficiency of phosphorothioate antisense increases in a near linear manner up to a final ethanol concentration of 50%, vol/vol. At an ethanol content greater than 50%, a large amount of aggregation/precipitation is observed. The effect of ethanol on vesicle formation can be further observed by monitoring both lipid and antisense loss on the filters during extrusion (Figure 4). At low ethanol contents, extrusion is slow and 15   the proportion of lipid and antisense loss is the same, suggesting that the losses are due to the formation of large complexes which get trapped on the filter. At ethanol contents of 30 and 40%, extrusion is very quick and losses of both lipid and antisense are minimal. As the ethanol content is increased above 40%, the loss of antisense becomes disproportionally high relative to the lipid. This can be attributed to the insolubility of DNA in high concentrations 20   of alcohol. Furthermore, in the presence of ethanol, PEG is required to prevent aggregation and fusion of the vesicles (results not shown).

EXAMPLE 2

          This example illustrates the effects of DODAP on the encapsulation of anti-25   sense, and further illustrates the effect of initial antisense concentration on the compositions.

          Having demonstrated that ethanol can greatly facilitate the preparation of lipid vesicles containing entrapped antisense, the next step was to examine the influence of DODAP (AL-1) content on the encapsulation of antisense (Figure 5). Accordingly, a 0.6 mL aliquot of a $[^3H]$-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with 0.4 mL of a 95% ethanol solution of lipid (DSPC:CHOL:DODAP: 30   PEG-CerC14; 100-(55+X):45:X:10, molar ratio) at final concentrations of 2 mg/mL and 9.9

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mg/mL, respectively. The molar ratio of DODAP was varied between 0 and 30%. The molar ratio of DSPC was adjusted to compensate for the changes in DODAP content. The samples were extruded ten times through three 100 nm filters as described in "Materials and Methods", and were dialyzed for 2-3 hours in 300 mM citrate buffer, pH 3.80, to remove a

5   majority of the excess ethanol. The samples were switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods". Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and

10   [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity. As seen in Figure 5, antisense encapsulation increased significantly between 5-20% DODAP. At DODAP contents greater than 20-25%, extrusion of the vesicles became more difficult suggesting the formation of complexes. At DODAP concentration of 40 and 50%, extrusion of the lipid / antisense mixture took hours compared to minutes for a

15   lipid composition containing 20% DODAP. To verify that the antisense was indeed associated with the lipid and that the observed encapsulation was not due to exchange of the [^{3}H]-label from the antisense onto the lipid, the antisense was extracted from the lipid using a Bligh and Dyer extraction. Using this technique, the antisense, which is soluble in the aqueous phase, was separated from the lipid (soluble in the organic phase) and quantified by

20   measuring the absorbance at 260 nm (Figure 6). While this method can underestimate the antisense concentration, the technique substantiated that the observed association of antisense with the lipid was not an artifact.

In yet another experiment, varying concentrations of a 20mer of [^{3}H]-phos-phorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) were mixed

25   with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio), 9.9 mg/mL (final concentration). The samples were extruded and dialyzed twice as described above. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods". Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and

30   [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and

- 40 -

[^{14}C]-lipid radioactivity. EPC:CH liposomes containing encapsulated antisense are included for comparison.

Optimization of the drug:lipid ratio was accomplished by increasing the initial antisense concentration that was mixed with 9.8 mg total lipid (DSPC:CHOL:DODAP:

5   PEG-CerC14; 25:45:20:10) (Figure 8). Drug:lipid ratios of up to 0.25, w/w, were obtained using 10 mg/mL of antisense in the preparation. However, the increased drug:lipid ratio was accompanied by a decrease in encapsulation efficiency, therefore a compromise must be made between optimizing the drug:lipid ratio and encapsulation efficiency. In comparison, antisense encapsulated by hydration of a dry lipid film (i.e. EPC:CHOL) in the absence of

10   cationic lipid typically yields low encapsulation efficiencies (< 12-15%) and drug:lipid ratios (< 0.1, w/w). Consequently, significant quantities of antisense are wasted during the encapsulation procedure.

EXAMPLE 3

15   This example illustrates the properties of the liposomal antisense formulations provided in the Materials and Methods above.

The size distribution of a liposomal preparation of antisense was determined by quasi-elastic light scattering (QELS) immediately after removal of the free antisense (A), and after storage of the preparation for 2 months at 4°C (B), using a Nicomp Model 370

20   sub-micron particle sizer. A 0.6 mL aliquot of a [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with 0.4 mL of a 95% ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The sample was extruded ten times through three 100 nm filters as described in "Materials and Methods", and dialyzed for

25   2-3 hours in 300 mM citrate buffer, pH 3.80, to remove a majority of the excess ethanol. The sample was switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods".

30   The size distribution and storage stability of antisense preparations described herein is demonstrated in Figure 7. The size distribution of a standard

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DSPC:CHOL:DODAP:PEG-CerC14 (25:45:20:10) preparation containing a 2 mg/mL initial antisense concentration was analyzed immediately after column chromatography to remove any free antisense. A very homogenous distribution is observed after preparation (119 ± 32 nm). This size distribution remained stable for at least 2 months after storage at 4°C (119 ± 32 nm).

5

EXAMPLE 4

This example illustrates the clearance pharmacokinetics, biodistribution and biological activity of an encapsulated murine ICAM-1 phosphorothioate antisense

10   oligodeoxynucleotide.

4.1   Plasma clearance

Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations

15   were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP:PEG-CerC14 (25:45:20:10), where X represents either distearoyl-phosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 µL) intravenously via the lateral tail vein of female

20   (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

The plasma clearance of three formulations, DSPC:CHOL:DODAP: PEG-CerC14, SM:CHOL:DODAP:PEG-CerC14, and POPC:CHOL:DODAP:PEG-CerC14,

25   of encapsulated antisense were examined in inflamed ICR mice (Figure 9). The circulation time was longest for the DSPC version of the formulation.

4.2   Organ accumulation

Liposomal antisense compositions were prepared and administered to mice as

30   outlined in the preceding section. Mice were terminated by cervical dislocation and the

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organs were recovered and processed as described in "Materials and Methods". Lipid and antisense recoveries were determined by standard scintillation counting techniques.

Organ accumulation of the various formulations was typical of previously described liposome clearance patterns, with the RES organs, principally the liver and spleen,

5   being responsible for the majority of clearance (Figure 10). One interesting observation is that the liver and spleen clearance account for only 40-45% of the total clearance of the "DSPC" formulation, suggesting that a significant population of vesicles is accumulating in another organ system or is being excreted.

10   *4.3   Stability.*

Liposomal antisense compositions were prepared and administered to mice as outlined in the preceding section. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques. Release rates were determined by measuring the [3H]/[14C] ratio over time.

15   The stability of the formulations was also assessed by measuring the ratio of antisense and lipid recovery in the blood at various times (Figure 11). A ratio of 1.0 suggests that the antisense and the lipid are staying together in the circulation. The "DSPC" formulation showed little deviation from a ratio of 1.0 over 24 h, suggesting that it is very stable in the circulation. The "POPC" formulation dropped to a ratio of 0.6 after 2 h, while

20   the ratio for the "SM" formulation decreased more slowly, reaching 0.6 after 12 h in the circulation. These results indicate that it may be possible to deliberately alter the antisense release rates by modifying the lipid composition.

4.4   PEG-Acyl Influence on circulation half-life of single dose of thioate antisense

25   Encapsulated lipid-encapsulated antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of DSPC:CHOL:DODAP:PEG-CerC14 or C20 (25:45:20:10). The formulation contained a lipid label ([14C]-cholesterylhexadecylether) and [3H]-antisense and were injected (200 μL)

30   intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120

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mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense

recoveries were determined by standard scintillation counting techniques.

The influence of PEG-acyl chain length on clearance rates of a

DSPC:CHOL:DODAP:PEG-Cer formulation was investigated using PEG-CerC14 and

5   PEG-CerC20 (**Figure 12**). The inclusion of PEG-CerC20 in the formulation resulted in

enhanced circulation times over the PEG-CerC14. This corresponds to in vitro data

suggesting that the C14 version of the PEG is exchanged much more rapidly out of the

vesicle than the C20 version.

10   *4.5*   *In vivo efficacy of single dose of lipid encapsulated ICAM-1 (phosphorothioate)*

antisense

The efficacy of PS- 3082 encapsulated in various lipid formulations containing

DODAP was tested in an ear inflammation model using ICR mice.

Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v.

15   dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped PS- 3082 (identified as

AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped PS- 3082 (identified as AS

4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped PS- 3082 (identified as AS

4200). Ear swelling was measured at 24 hours after initiating inflammation using an

engineer's micrometer.

20   Ear swelling measurements were made 24 hours after initiating inflammation

in mice treated i.v. at the time of ear challenge with either HBS (control), PS- 3082

encapsulated in EPC:CHOL vesicles (30 mg/kg dose of oligo), PS- 3082 encapsulated in

POPC:CHOL:DODAP:PEG-CerC14 vesicles (30 mg/kg dose of oligo), or PS- 3082

encapsulated in DSPC:CHOL:DODAP:PEG-CerC14 vesicles (30 mg/kg dose of oligo)

25   (**Figure 13**). The "DSPC" formulation resulted in the greatest efficacy, exhibiting only 10%

increase in ear swelling over pre-challenge values. A similar trend was observed for cellular

infiltration into the "challenged" ear versus the non-treated ear (**Figure 14**).

In another evaluation, mice received 10 μCi of [^{3}H]-methylthymidine, i.p., 24

hours before initiating inflammation. Inflamed mice were treated at the time of ear challenge

30   with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped

PS- 3082 (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped PS-

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3082 (identified as AS 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped PS-3082 (identified as AS 4200). Cell infiltration was monitored by measuring the radioactivity in the "challenged ear" versus the non-treated ear. Results are expressed as the ratio of radioactivity in the left (challenged ear) versus right ear.

5

4.6   In vivo efficacy of single dose of lipid encapsulated ICAM-1 (phosphodiester) antisense

This experiment demonstrates the *in vivo* efficacy of a phosphodiester antisense oligodeoxynucleotide encapsulated in lipid particles according to the invention. In specific,
10   the phosphodiester was targeted to the ICAM-1 gene in an ear inflammation model.

Group	Test Sample/Drug	Dose	Time Point
1	control inflammation - HBS	200 μl	24 hr
2	corticosteroid	200 μl	24 hr
3	empty vesicles	200 μl	24 hr
4	PS-3082	200 μl	24 hr
5	PO-3082	200 μl	24 hr

Antisense Sample Preparation: Antisense was encapsulated using the standard methods of Examples 5-9, using the phosphodiester modification. The phosphodiester formulation
20   used 10-50 mM citrate (preferably 20 mM citrate), pH 4.0 instead of 300 mM citrate, pH 4.0 preferred for phosphorothioates. Empty vesicles consisted of lipid components only. Corticosteroid (either Halobetasol propionate 0.05% by weight (Westwood Squibb, Montreal) or Dexamethasone (50 ug dissolved in 4:1 acetone:olive oil)) was applied topically in a thin film to cover the surface of the ear 15 minutes after ear challenge.

25   **Inflammation and Dosing:** Mouse ear inflammation was induced using DNFB as described above in Materials and Methods. Female ICR mice (6-8 weeks old) received intravenous tail vein injections of antisense (200 μl). Antisense doses for the phosphorothioate and phosphodiester antisense were adjusted to be 20-30 mg/kg. 6 mice were tested with each formulation. Administration occurred 15 min. after the application of 0.2% DNFB to the
30   mouse ear. Ear measurements were made on anaesthetized mice 24 hours after treatment (unless shown otherwise) and prior to termination. Mice are terminated by cervical dislocation and the ears are removed around the pinna. Ears are then weighted, digested

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(Solvable) and analyzed for radioactivity by liquid scintillation counting. Ears were analyzed

for 1) Ear edema - based on the increase in ear thickness due to ear swelling. Calculated by

subtracting pre-ear thickness values from post-ear thickness values **Figure 21**. 2) Cell

infiltration - based on radioactivity accumulated in the inflamed (right) ear vs. the control

5   (left) ear **Figure 22**; and 3) Ear weights – left ear versus right ear (measurement of edema)

Figure 23.

Results: The controls consisting of buffer alone (HBS) or Empty Vesicles alone

demonstrated no efficacity. Topical corticosteroid demonstrates its known excellent

efficacity by reducing inflammation to below pre-challenge levels. Both the

10   phosphorothioate and phosphodiester antisense show excellent efficacy through a systemic

delivery administration, reducing the degree of inflammation by around 70% and 85%,

respectively. Thus, it is possible to administer the compositions of the invention at a site

where the disease site is distal to the site of the injection.

15   4.7   *In vivo efficacy of US3 antisense (Tumor Window Model)*

In this example, the anti-tumor activity of lipid encapsulated US3, an antisense

oligonucleotide directed at the erb-B-2 gene, has been demonstrated in an *in vivo* human

breast tumor model.

The human breast carcinoma line MDA-MB-453 was implanted in a mouse

20   tumor window model according to the method of Wu, N.Z., Da, D., Rudoll, T.L., Needham,

D., Whorton, R. & Dewhirst, M.W. 1993. Increased microvascular permeability contributes

to preferential accumulation of Stealth liposomes in tumor tissue. Cancer Research 53: 3765-

3770; and Dewhirst, M.W., Tso, C.Y., Oliver, R., Gustafson, C.S., Secomb, T.W. & Gross,

J.F. 1989. Morpholigic and hemodynamic comparison of tumor and healing normal tissue

25   microvasculature. Int. J. Radiat. Oncol. Biol. Phys. 17: 91-99. See also Dewhirst, MW., and

Needham, D. 1995. Extravasation of Stealth Liposomes into Tumors: Direct Measurement of

Accumulation and Vascular Permeability using a Skin Flap Window Chamber. In Stealth

Liposomes (Eds. Lasic, D. and Martin, F.) CRC Press.

The lipid-antisense formulation consists of disteroylphosphatidylcholine

30   (DSPC, 25 mol%), cholesterol (Chol, 45 mol%), dioleoylphosphatidyldiaminopropane,

(DODAP, or AL1, 20 mol%) and PEG-ceramide (C14 chain length, 10 mol%). For some

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experiments detailed below, proportions and constituents were altered, but the method of
preparation remained the same. Lipids were dissolved in ethanol at 20 mg/ml (PEG-ceramide
at 50 mg/ml). Routinely, 1 to 2 μCi ^{14}C-cholesterylhexadecylether was added as a lipid
radiolabel. Lipids were mixed in the correct proportions in ethanol to a final concentration of

5   10 mg in 400 μl. The lipid mixture was then added dropwise to phosphorothioated antisense
(US3: anti-human erb-B-2 GGT GCT CAC TGC GGC (SEQ ID. No 3) dissolved in 300 mM
citrate buffer pH 4.0 (600 μl to make a final volume of 1 ml). The antisense was used at a
variety of concentrations, but the optimum concentration for maximum encapsulation
efficiency and drug:lipid ratio was determined to be 0.5 mg/ml final. During the addition, the

10   solution becomes opaque. The DODAP is positively charged at pH 4.0 (pKa = 6.53) and so
attracts the negatively charged DNA molecules. The mixture was subjected to five cycles of
freezing in liquid N_2 and thawing at 65 °C followed by extrusion through 100 nm filters ten
times at 65 °C.

          After extrusion, two methods can be used for removal of the external

15   antisense. Firstly, the liposomes are diluted 2:1 with citrate (to reduce ethanol content to
20%) then applied to a Bio-Gel A18M 100-200 mesh column equilibrated with HBS. The
column profiles shown in this report were generated in this manner. Alternatively, the
liposomes are dialysed 2h against citrate to remove ethanol, the overnight against HBS to
increase the external pH. The resulting mixture is then applied to a DEAE cation exchange

20   column to remove external oligo. This method was the routine method used for sample
preparation for *in vivo* studies. Antisense concentrations were routinely determined by A260
measurements. Lipid concentrations were determined by scintillation counting after spiking
initial mixture with a known concentration of ^{3}H or ^{14}C cholesterylhexadecyl ether, or by
HPLC. Encapsulation efficiency was determined by division of the final drug to lipid ratio

25   by the initial drug to lipid ratio.

          In vivo efficacy evaluation: When the tumor in the window has reached a
diameter of 2-3 mm, treatment with free or TCS-encapsulated US3 oligonucleotide is
initiated. Treatment consists of a 200 ul intravenous administration (tail vein) of either free
US3 or TCS-encapsulated US3 on a 3 administrations/week schedule and an antisense dose

30   of 10 mg/kg/administration. Tumor size is monitored 3 times per week by microscopy.

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Results: The TCS-encapsulated US3 oligonucleotide was very effective at preventing the growth, or causing extensive size reduction, of the MDA-MB-453 human breast carcinoma in the window model. In contrast, unencapsulated oligonucleotide was ineffective at inhibiting tumor growth.

5

4.8   *In vivo clearance of various formulations using alternative amino lipids: DODAP or DODMA*

Antisense particle formulations were prepared according to Example 2, with the following modifications: In assay#1 and #2, 25% AL-1 (hydrochloride salt of DODAP)
10   and 25% free base DODAP were employed, respectively, with a concomitant reduction in the amount of DSPC. Assay#3, 4 and 5 employed 30%, 25% and 20% DODMA (free base (prepared at Inex Pharmaceuticals Corp., Burnaby BC)), respectively, again with a concomitant reduction of DSPC.

Both the encapsulation efficiency and *in vivo* clearance of the formulations
15   were studied. There was no significant difference between the encapsulation or clearance of the free base or HCl salt of DODAP. Decreasing DODMA concentration (30, 25, 20 %) severely decreased the encapsulation efficiency of PS-2302 (91%, 43%, 35%) and likewise the Drug/Lipid ratio of the resulting formulation.

In the clearance study outlined in **Figure 16**, DODMA formulations
20   demonstrated slightly higher rates of clearance than 25 % DODAP or AL-1, although all formulations appear to be retained in the circulation to a degree which is suitable for human therapeutics.

4.9   *PEG-acyl influence on clearance rate of repeat doses of encapsulated EGF-R*
25   *phosphorothioate antisense*

Lipid -encapsulated antisense was prepared using the ethanol-citrate procedure as described above, with changes to molar ratios of components as indicated. Initial lipid and antisense concentrations were about 9.9 and 2 mg/mL, respectively. DODAP containing formulations had drug:lipid ratios of 0.15 (+/-) 0.05. Passive encapsulation systems had
30   drug:lipid ratios of 0.03. Nine different liposomal formulations were prepared, using standard techniques, in the following molar ratios:

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Formu-lation	DSPC (mol%)	Chol (mol%)	DODAP (mol%)	Steric Barrier Derivatized Lipid (name: mol%)	Antisense (EGF-R 2mg/ml)
1	55	45	Nil	Nil	Empty
2	50	45	Nil	ATTA8-DSPE : 5	Empty
3	50	45	Nil	ATTA8-DSPE : 5	AS
4	20	45	30	ATTA8-DSPE : 5	AS
5	20	45	30	PEG-DSPE : 5	AS
6	25	45	25	PEG-CerC14 : 5	Empty
7	25	45	25	PEG-CerC14 : 5	AS
8	25	45	25	PEG-CerC20 : 5	Empty
9	25	45	25	PEG-CerC20 : 5	AS

Antisense ("AS") used was fully phosphorothioated EGFR (anti-human Epidermal Growth Factor Receptor) CCG TGG TCA TGC TCC (SEQ ID. No 10) (prepared by Hybridon, Inc.)

PEG-CerC14 is PEG(mw2000)-Ceramide with 14 carbon acyl chain.

PEG-CerC20 is PEG(mw2000)-Ceramide with 20 carbon acyl chain.

PEG-DSPE is PEG(mw2000)- 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine

ATTA8-DSPE is N-(ω-N'-acetoxy-octa(14'amino-3',6',9',12'-tetraoxatetradecanoyl))-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (molec weight about 2660). Synthesis of ATTA8-DSPE is fully disclosed in US Provisional Pat. Application Serial No. 60/073,852, filed 23 - Dec – 1997 and US Provisional Pat. Application filed 2-Feb-1998 (Attorney Docket No.: TT&C 16303-005810) both assigned to the assignee of the instant invention and incorporated herein by reference.

Each formulation contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense, as described in Example 4.4, above. All samples were prepared in 300 mM citrate pH 4.0 containing 40% ethanol and extruded 10X through 100 nm filters. Formulations contained phosphorothioate antisense and lipid or empty lipid alone. Samples were dialyzed in HBS (20 mM Hepes, 145 mM NaCl, pH 7.45) to remove ethanol and citrate. Sample lipid concentrations were adjusted such that the injected lipid dose will be 1.8 μmol/mouse/week (5-10 mg AS per kg mouse/week). Samples were filtered (0.22 μm) prior to injection.

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In this experiment female (20-25g) ICR mice (6-8 weeks old) were divided into 9 groups of 6, plus other control groups. Each group received four injections of the same formulation. All injections were 200 µL intravenous (via the lateral tail vein) at a lipid dose of 120 mg/kg. Mice were dosed every week for 3 weeks (4 injections). At 4 weeks, certain groups (treated with lipid and antisense) were given an injection of empty lipid carriers of varying composition to evaluate whether there is rapid clearance of the carrier in the absence of antisense. Blood (25 μl, pipettor) was collected 1 h post-injection each week for 3 weeks by tail nicks. Mice were weighed each week to estimate blood volume (8.0 ml whole blood/100 g body weight). Blood was placed in a glass scintillation vial containing 200 μl of 5% EDTA. Solvable (500 μl) was added and the blood was digested for 3 h at 65°C. Samples were decolorized by the addition of 100 μl 70% hydrogen peroxide. Samples were analyzed for radioactivity by liquid scintillation counting. At the end of 4 weeks, mice were terminated by CO_2 inhalation or cervical dislocation preceded by general anesthesia.

The results of this experiment are shown in **Figure 17.** For all formulations not containing antisense ("empty liposomes") repeat dosages demonstrated circulation times reasonably consistent with the first dosage. However, when antisense is used in the formulation, it was surprisingly found that the acyl chain length of the lipid derivatized to the steric barrier (i.e. ATTA or PEG) moiety demonstrates a profound effect on clearance rates. Repeat dosages of PEG-CerC20, PEG-DSPE and ATTA8-DSPE formulations are rapidly cleared from the circulation compared to the first dosage, whereas the PEG-CerC14 formulation is reasonably consistent with the first dosage.

Similar results are demonstrated in **Figure 18**. The formulations were identical to those of **Figure 17**, with the additional formulation of empty vesicles using the same lipids as formulations 4 and 5.

Without intending to be bound by any particular theory of action, it is suggested by these results that lipids like the PEG-CerC14 lipid, a lipid which exchanges out of the liposome membrane with a T1/2 on the order of minutes (i.e. 1-60mins) in blood provides a tremendous benefit over lipids like PEG-CerC20, PEG-DSPE and ATTA8-DSPE which do not exchange out, where repeat dosing of a lipid-formulated compound, such as a therapeutic compound or diagnostic compound, is required. The mammalian blood clearance response may not recognize these as foreign antigens if the derivatized lipid is removed

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expeditiously from the liposome surface when in circulation. However, when the derivatized-lipid remains with the formulation for extended periods, a clearance response is invoked, which causes rapid clearance upon repeat dosing. This data suggests that any lipid derivatized with a steric barrier molecule that exchanges out of the liposome membrane faster

5   than PEG-CerC20, PEG-DSPE or ATTA8-DSPE will be superior for use in repeat dosing. For example ATTA8-DMPE, or PEG-CerC8 to C18 all being exchangeable, will have improved circulation characteristics upon repeat administration.

Taken together, it will be evident to one skilled in the art, that on the basis of these teachings, any diagnostic or therapeutic agent that may be delivered in a lipid

10   formulation comprising a steric-barrier derivatized lipid, such as a PEG-lipid or ATTA-lipid, should be tested with both a long and short acyl-chain anchors, in order to determine which formulation is best for repeat dosings.

Further, without intending to be bound by any theory of action, the invention herein may prove to be particularly useful when the bioactive agent being delivered is a non-

15   cytotoxic agent. Cytotoxic agents kill those cells which clear long circulating (i.e. PEG-DSPE) liposomes. This ensures that repeat dosings will not be rapidly cleared, because the cells responsible (usually macrophages) do not survive. In these situations, the acyl-chain length may not be significant. However, where the bioactive agent is non-cytotoxic, such as in the case of antisense drugs (regardless of chemistry or target), plasmids, proteins, etc., and

20   many conventional drugs, the invention will be useful for repeat dosing.

4.10   In vivo efficacy of repeat doses of encapsulated phosphorothioate c-myc antisense in an oncology model.

In vivo efficacy of repeat injections of using formulations of the invention are

25   shown in a mouse tumor system in **Figure 19.** This experiment demonstrated efficacy of the antisense formulated according to the invention in a human oncology model, and showed the importance of PEG-acyl chain length on the efficacy of repeat dosings.

Lipid-antisense particle formulation: Formulations were prepared as described in these Examples.

30

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Formulation	DSPC (mol%)	Chol (mol%)	DODAP (mol%)	Steric Barrier Derivatized Lipid (name: mol%)	Antisense (c-myc 2mg/ml)
HBS Buffer					Empty
AS4200 (c-myc)	25	45	25	PEG-CerC14 : 5	LR-3280
AS4204 (c-myc)	25	45	25	PEG-CerC20 : 5	LR-3280
AS4204 (c-myc SCR)	25	45	25	PEG-CerC20 : 5	c-myc SCR
AS4204 (PS-2302)	25	45	25	PEG-CerC20 : 5	PS-2302
AS4204(PS-3082)	25	45	25	PEG-CerC20 : 5	PS-3208
c-myc					LR-3280
c-myc SCR					c-myc SCR
PS-2302					PS-2302
PS-3082					PS-3082
AS4200 (no antisense)	25	45	25	PEG-CerC14 : 5	Empty
AS4204 (no antisense)	25	45	25	PEG-CerC20 : 5	Empty

Antisense used were:

LR-3280:	human c-myc gene (phosphorothioate)	
	AAC GTT GAG GGG CAT	(SEQ ID. No 4)
c-myc SCR:	GAA CGG AGA CGG TTT	(SEQ ID. No 17)
PS-2302	human ICAM-1 (phosphorothioate)	
	GCCCAAGCTGGCATCCGTCA	(SEQ ID. No 2)
PS-3082	murine ICAM-1 (Intracellular Adhesion Molecule-1) (phosphorothioate)	
	TGCATCCCCCAGGCCACCAT	(SEQ ID. No 1)

Formulations were diluted in filtered HBS, pH 7.6 to achieve required antisense dose (i.e. lipid dose decreases as well). Samples were filtered (0.22 μm) prior to injection. External buffer was HBS (20 mM Hepes, 145 mM NaCl, pH 7.6). Free antisense

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was dissolved in HBS and adjusted to the required dose by A260 (Extinction coefficients: active and control c-myc = 30.6, PS-2302 = 32.8, PS-3082 = 33.6).

Tumour Inoculum: B16/BL6 murine melanoma cells were maintained in culture in MEM media supplemented with 10% FBS. On day 0 of the study, 3×10^5 cells were injected sub-
5 cutaneously (s.c.) into the dorsal flank (injection volume: 50 μl) of female C57BL/6 mice (20-23 g). Typically, 15% extra mice will be injected so non-spheroidal tumours or mice in which no tumours are observed can be excluded from the study. Tumours were allowed to grow for a period of 5-7 days until tumors reached 50-100 mm³ prior to initiating treatments with test samples/controls.

10 **Treatment:** On the day of first treatment mice with acceptable tumours were randomly grouped with 5 animals per group. Treatment began when tumours were 50-100 mm³. Mice were dosed every other day for a total of 7 doses. Administrations were via intravenous tail vein injections (200 ul). Initial drug:lipid ratio of formulation was 0.20 (w/w) and the final drug:lipid ratio (0.14) was held constant; consequently, the lipid concentration varied as
15 samples were diluted to the desired antisense concentration. The antisense dose was 10 mg/kg.

Endpoints: Primary tumour volume was measured using calipers. Length (mm) and width (mm) measurements were made every other day (on non-injection days) for the duration of the study. Tumour height measurements (mm) were made when feasible. Tumour volumes
20 were calculated using the following formulas:

#1   Tumour Volume (mm³) = $(L \times W^2)/2$

#2   Tumour Volume (mm³) = $(L \times W \times H) \times \pi/6$

Mice were euthanized when tumour volumes reach 10% of body weight or on the first signs of ulceration. Mouse weights were recorded every day during the dosing portion of the study.
25 On termination, all tumours were excised, weighed, observed by FACS analysis or by Northern/Western analysis. Mice were euthanized by CO_2 inhalation or cervical dislocation preceded by general anesthesia.

Results: Figure 9 shows weights of tumors excised and weighed at day 18 for all groups treated with antisense at 10 mg/kg/dose compared with empty lipid controls. Tumour sizes
30 for the AS4200(c-myc) group exhibited the best efficacy and were very consistent with only small ranges in tumour volumes observed (285-451 mm³). The group treated with free c-myc

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also resulted in smaller tumours but exhibited more variability in tumour volume (156-838 mm³). The encapsulated c-myc controls (c-myc SCR/PS-2302/PS-3082), AS4204(c-myc), empty lipid carriers, and free antisense controls, however, showed no inhibitory effect on tumor volumes over the 18 days when compared to HBS controls.

5   c-myc expression in tumor tissue was also evaluated by FACS. A correlation between tumour size and c-myc protein expression was detected (data not shown).

To determine the importance of the stability of the PEG-polymers, PEG-acyl chain length was evaluated using formulations containing PEG-CerC14 and PEG-CerC20. Interestingly, the formulation containing the PEG-CerC20 (AS4204) showed no apparent

10   efficacy at any of the doses studied. The PEG-CerC14 formulation (AS4200) showed a dose response. The difference observed between the PEG-CerC14 and PEG-CerC20 formulations may reflect the rapid clearance phenomenon that has been observed in other models.

To establish the tolerability of free and encapsulated antisense, mouse weights were measured on a daily basis during the treatment phase of the study. No significant

15   changes in mouse weights for either free or encapsulated formulations were apparent over the course of the dosing phase or throughout the study.

EXAMPLE 5

This example illustrates a high efficiency formulation according to Example 2,

20   but instead of phosphorothioate antisense, employing 1) a phosphodiester antisense compound having exclusively phosphodiester internucleotide linkages (PO-2302 anti-human ICAM-1 GCCCAAGCTGGCATCCGTCA (SEQ ID. No 1)) prepared by Inex Pharmaceuticals (USA), Inc., Hayward CA) or 2) ribozyme molecule to VEGF-R-1 (human Vascular Endothelial Growth Factor Receptor 1) comprising a modified RNA sequence of

25   GAG UUG CUG AUG AGG CCG AAA GGC CGA AAG UCU G (SEQ ID. No 16).

A 15mer of [³H]-phosphodiester antisense oligodeoxynucleotide (PO-2302) in citrate buffer, pH 3.80 (experiments ranged from 10-1000 mM citrate) was mixed with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The final ethanol

30   concentration in the 1 ml preparation was 38% vol/vol. The sample was extruded ten times through three 100 nm filters as described in "Materials and Methods". The sample was

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dialyzed for 2-3 hours in citrate buffer, pH 3.80 (same molarity as experiment), to remove a majority of the excess ethanol. The samples were switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. Non-encapsulated antisense was removed either by this regular dialysis, tangential

5   flow dialysis, or chromatography. Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

Figure 15 illustrates results. Encapsulation efficiency was over 50% across the 10-50 mM citrate range, and all final (administration ready) drug:lipid ratios were greater

10   than 10% by weight. Parallel experiments varying citrate concentration were conducted with phosphorothioate antisense PS-2302. Results are also above 50% encapsulation, and in fact show a higher encapsulation efficiency than phosphodiesters, particularly at higher citrate concentrations.

This experiment was repeated using 20mM citrate instead of 300 mM citrate

15   to encapsulate the ribozyme molecule to VEGF-R-1 (human Vascular Endothelial Growth Factor Receptor 1) GAG UUG CUG AUG AGG CCG AAA GGC CGA AAG UCU G (SEQ ID. No 16). **Figure 20** shows the encapsulation efficiency of the ribozyme at was over 50%, approximately the same as the phosphodiester.

20   **EXAMPLE 6**

This example illustrates a high efficiency formulation as in Example 5, but replacing DODAP with an alternative protonatable lipid. Typically, the preparation for the alternative will be X:DSPC:CHOL:PEG-CerC14 at 20:25:45:10 molar ratio where X can be DODAC, OA, DODMA or any other lipid suitable for the invention.

25   Materials: distearoylphosphatidylcholine, DSPC; cholesterol, CHOL (both from Northern Lipids, Vancouver, BC); N,N-dioleyl-N,N-dimethylammonium chloride, DODAC; Oleylamine, OA (prepared by Steve Ansell, Inex); N-(1-(2,3-Dioleoyloxy) propyl)-N,N,-dimethyl ammonium chloride, DODMA(Avanti Polar Lipids, Alabaster AB, chloride salt prepared by Steve Ansell, INEX); poly(ethylene glycol)2000 coupled to a

30   ceramide derivative with 14 carbon acyl chains, PEG-CerC14 (Zhou Wang, INEX Pharmaceuticals); 13 x 100 mm glass tube; filter sterilized 300 mM citrate buffer, pH 3.9 -

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4.0 (use a 0.2 μm filter). Fully thioated c-myc antisense (INEX (USA), Hayward Ca),

Anhydrous Ethanol (Commercial Alcohols, Toronto, On), Citric acid, Monobasic Sodium

phosphate, Dibasic Sodium phosphate, Sodium hydroxide, HEPES (BDH, Mississauga On).

Deionized water, Chloroform, Methanol, Oligreen™ oligonucleotide reagent (Molecular

5   Probes, Eugene Or), Sodium chloride, Triton X-100, alcohol dehydrogenase reagent kit,

(Sigma Chemical Co., St Louis Mo.),

Lipid stock solutions were made in 100 % ethanol with the working

concentrations of the lipids which is as follows:

DSPC, 20 mg/ml; CHOL, 20 mg/ml (not very soluble above this concentration);

10   DODMA, 20 mg/ml; PEG-CerC14; 50 mg/ml.

To prepare stock solutions of antisense, the antisense molecules were

dissolved in the filtered 300 mM citrate buffer at a concentration of 3.33 mg/ml. Lipids were

mixed in the desired proportions in a 13 x 100 mm glass tube to achieve a final volume of 0.4

ml of lipids using 100 % ethanol as listed in table 1, below:

15

Table 1. Proportional mixture of lipids in a 13 x 100 mm glass test tube.

Lipid	Mol %	M. Wt.	mg	μmol	Stock (mg/ml)	Vol of Stock (μl)
DODMA	20	652.6	1.69	2.60	20	84.5
DSPC	25	790	2.57	3.25	20	115
CHOL	45	386.7	2.26	5.85	20	113.1
PEG-CerC14	10	2600	3.38	1.30	50	67.6
	100		9.9	13.00		380.2

25   In a separate 13 x 100 mm glass tube, 0.6 ml of antisense at 3.33 mg/ml was added. The

pH of this solution should be 3.9 - 4.0. (*NOTE:* the antisense concentration is <u>NOT</u> determined

by weight but rather by measuring absorbance at 260 nm). The lipid mixture solution was

warmed to 65°C for about 2 minutes. The antisense tube was vortexed and during this time,

using a Pasteur pipette, the lipids (in ethanol) were added slowly in a dropwise manner. The

30   mixture will get "cloudy" and some bubbles may be observed due to the ethanol, but no

aggregates should be present. The resulting volume of the antisense-lipid mixture was 1.0 ml

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with a 10 mg (13 umol) total lipid at 13 μmols, 2 mg of antisense, and 38 % ethanol, vol/vol. It can be expected that the pH to rise to about 4.4.

The antisense-lipid mixture was subjected to 5 (five) cycles of freezing in liquid nitrogen and thawing at 65°C in a waterbath. After each thaw, the mixture was vortexed briefly. Subsequently, the mixture was passed 10 times through three stacked 100 nm polytcarbonate filters (Poretics) or extruded using a pressurized extruder apparatus with a thermobarrel attachment (Lipex Biomembranes). The temperature and nitrogen pressure during extrusion were 65°C and no more than 200 psi to 300 psi, respectively. Each pass should take no more than 2 minutes and is vortexed after each pass.

After extrusion, the mixture was dialyzed in a dialysis tubing (3500 Mwt cutoff; SpectraPor) for 1 hour in 300 mM citrate at pH 3.9-4.0, removing the ethanol. The mixture was transferred into 5 L of HBS buffer at pH 7.5 and allowed to further dialyze to a minimum of 12 hours, to neutralize the DODMA and release any surface bound antisense associated with the vesicles. Alternatively, tangential flow dialysis, ion exchange-chromatography or gel filtration chromatography can be used to process the extruded antisense-lipid mixture to an administration ready preparation.

EXAMPLE 7

This example illustrates a high efficiency formulation as in Example 5, but replacing DSPC with SM to generate a preparation of DODAP:SM:CHOL:PEG-CerC14 at 20:25:45:10 molar ratio. Antisense is processed with the formulation for a standard 1.0 ml volume, which can be scaled up proportionately as required.

Materials: Sphingomyelin SM; cholesterol, CHOL; dimethylaminopropane, DODAP; polyethylene glycol coupled to a ceramide derivative with 14 carbon acyl chains, PEG-CerC14; 13 x 100 mm glass tube; filter sterilized 300 mM citrate buffer, pH 3.9 - 4.0 (use a 0.2 μm filter).

Lipid stock solutions were made in 100 % ethanol with the working concentrations of the lipids which is as follows:

SM, 20 mg/ml; CHOL, 20 mg/ml (not very soluble above this concentration); DODAP, 20 mg/ml; PEG-CerC14; 50 mg/ml.

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To prepare stock solutions of antisense, the antisense molecules were dissolved in the filtered 300 mM citrate buffer at a concentration of 3.33 mg/ml. Lipids were mixed in the desired proportions in a 13 x 100 mm glass tube to achieve a final volume of 0.4 ml of lipids using 100 % ethanol as listed in Table 2, below:

5 **Table 2. Proportional mixture of lipids in a 13 x 100 mm glass test tube.**

Lipid	Mol %	M. Wt.	mg	µmol	Stock (mg/ml)	Vol of Stock (µl)
DODAP	20	684.5	1.78	2.60	20	89.0
SM	25	703	2.30	3.27	20	115
CHOL	45	386.7	2.26	5.85	20	113.1
PEG-CerC14	10	2600	3.38	1.30	50	67.6
	100		9.72	13.02		384.7

In a separate 13 x 100 mm glass tube, 0.6 ml of antisense at 3.33 mg/ml was added. The pH of this solution should be 3.9 - 4.0. (*NOTE:* the antisense concentration is NOT determined by weight but rather by measuring absorbance at 260nm). The lipid mixture solution was warmed to 65°C for about 2 minutes. The antisense tube was vortexed and during this time, using a Pasteur pipette, the lipids (in ethanol) were added slowly in a dropwise manner. The mixture will get "cloudy" and some bubbles may be observed due to the ethanol, but no aggregates should be present. The resulting volume of the antisense-lipid mixture was 1.0 ml with a 10 mg (13 umol) total lipid at 13 µmols, 2 mg of antisense, and 38 % ethanol, vol/vol. It can be expected that the pH to rise to about 4.4.

The antisense-lipid mixture was subjected to 5 (five) cycles of freezing in liquid nitrogen and thawing at 65°C in a waterbath. After each thaw, the mixture was vortexed briefly. Subsequently, the mixture was passed 10 times through three stacked 100 nm polytcarbonate filters (Poretics) or extruded using a pressurized extruder apparatus with a thermobarrel attachment (Lipex Biomembranes). The temperature and nitrogen pressure during extrusion were 65°C and no more than 200 psi to 300 psi, respectively. Each pass should take no more than 2 minutes and is vortexed after each pass.

After extrusion, the mixture was dialyzed in a dialysis tubing (3500 Mwt cutoff; SpectraPor) for 1 hour in 300 mM citrate at pH 3.9-4.0, removing the ethanol. The mixture was transferred into 5 L of HBS buffer at pH 7.5 and allowed to further dialyze to a minimum of 12

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hours, to neutralize the DODAP and release any surface bound antisense associated with the vesicles. Alternatively, tangential flow dialysis, ion exchange-chromatography or gel filtration chromatography can be used to process the extruded antisense-lipid mixture to an administration ready preparation.

5

EXAMPLE 8

This example illustrates a high efficiency formulation as in Example 5, but replacing PEG-CerC14 with ATTA8-DSPE to prepare DODAP:DSPC:CHOL:ATTA8-DSPE at 40:10:45:5 molar ratio of antisense formulation.

10   Materials: distearoylphosphatidylcholine, DSPC; cholesterol, CHOL; dimethylaminopropane, DODAP; N-(ω-N'-acetoxy-octa(14'amino-3',6',9',12'-tetraoxatetradecanoyl))-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, ATTA8-DSPE; 13 x 100 mm glass tube; filter sterilized 300 mM citrate buffer, pH 3.9 - 4.0 (use a 0.2 μm filter).

Lipid stock solutions were made in 100 % ethanol with the working

15   concentrations of the lipids which is as follows:

DSPC, 20 mg/ml; CHOL, 20 mg/ml (not very soluble above this concentration); DODAP, 20 mg/ml; ATTA8-DSPE; 50 mg/ml.

To prepare stock solutions of antisense, the antisense molecules were dissolved in the filtered 300 mM citrate buffer at a concentration of 3.33 mg/ml. Lipids were mixed in the

20   desired proportions in a 13 x 100 mm glass tube to achieve a final volume of 0.4 ml of lipids using 100 % ethanol as listed in Table 3, below:

Table 3. Proportional mixture of lipids in a 13 x 100 mm glass test tube.

Lipid	Mol %	M. Wt.	mg	μmol	Stock (mg/ml)	Vol of Stock (μl)
DODAP	40	684.5	4.16	6.08	20	208
DSPC	10	790	1.2	1.52	20	60
CHOL	45	386.7	2.6	6.72	20	130
ATTA8-DSPE	5	2638	2.0	0.76	50	40
	100		10.26	15.1		438

30

In a separate 13 x 100 mm glass tube, 0.6 ml of antisense at 3.33 mg/ml was added. The pH of this solution should be 3.9 - 4.0. (*NOTE:* the antisense concentration is NOT determined

- 59 -

by weight but rather by measuring the absorbance at 260nm). The lipid mixture solution was warmed to 65°C for about 2 minutes. The antisense tube was vortexed and during this time, using a Pasteur pipette, the lipids (in ethanol) were added slowly in a dropwise manner. The mixture will get "cloudy" and some bubbles may be observed due to the ethanol, but no

5 aggregates should be present. The resulting volume of the antisense-lipid mixture was 1.0 ml with a 10 mg (13 umol) total lipid at 13 μmols, 2 mg of antisense, and 38 % ethanol, vol/vol. It can be expected that the pH to rise to about 4.4.

The antisense-lipid mixture was subjected to 5 (five) cycles of freezing in liquid nitrogen and thawing at 65°C in a waterbath. After each thaw, the mixture was vortexed briefly.

10 Subsequently, the mixture was passed 10 times through three stacked 100 nm polytcarbonate filters (Poretics) or extruded using a pressurized extruder apparatus with a thermobarrel attachment (Lipex Biomembranes). The temperature and nitrogen pressure during extrusion were 65°C and no more than 200 psi to 300 psi, respectively. Each pass should take no more than 2 minutes and is vortexed after each pass.

15 After extrusion, the mixture was dialyzed in a dialysis tubing (3500 Mwt cutoff; SpectraPor) for 1 hour in 300 mM citrate at pH 3.9-4.0, removing the ethanol. The mixture was transferred into 5 L of HBS buffer at pH 7.5 and allowed to further dialyze to a minimum of 12 hours, to neutralize the DODAP and release any surface bound antisense associated with the vesicles. Alternatively, tangential flow dialysis, ion exchange-chromatography or gel filtration

20 chromatography can be used to process the extruded antisense-lipid mixture to an administration ready preparation.

EXAMPLE 9

This example illustrates use of tangential flow dialysis to clean up a large scale

25 (>50 ml) preparation of extruded antisense-lipid mixture to obtain an administration ready preparation. Tangential Flow Diafiltration has been shown to be useful in four functions in the formulation process 1) buffer exchange, 2) removal of ethanol, 3) removal of unencapsulated antisense and 4) concentration of the formulation. Using TF it is demonstrated that it is possible to efficiently exchange these components using only 10-15 sample volumes with a single buffer

30 system at a very significant reduction in the process time.

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Materials for Tangential Flow Dialysis: Microcross Sampler™ Tangential Flow column (Microgon, Laguna Hills, Ca) Masterflex™ console drive and Easyload™ Pump head (Cole-Parmer, Vernon Hills Ill.), Extruder (Lipex Biomembranes, Vancouver BC), Polycarbonate membranes, 100 µm, (AMD Manufacturing, Mississauga On).

5   Antisense (c-myc) is prepared by dissolving in 300 mM Na Citrate buffer to a final concentration of 4.17 mg/ml for c-myc as verified by absorbance at 260 nm. The antisense stock solution is typically warmed to 65°C for 2 minutes to dissolve and to remove secondary structure. AS4200 consists of DODAP:DSPC:CHOL:PEG-CER-14 at the percent mol ratio of 25:20:45:10 and the lipids are aliquoted from stock solutions to a total concentration of 10

10   mg/0.400 ml in anhydrous ethanol. In this study 50 - 60 ml scale formulations were produced. Thus 20-24 ml of the ethanolic lipid solution is added dropwise, at room temperature, using a peristaltic pump at 1ml/nin into 30 - 36 ml of the AS solution which is stirring in a 100 ml round bottom flask with a 2 cm magnet stir bar (Stirrer setting 2-3). After mixing, the lipid/antisense suspension was pipetted into a 100 ml extruder prepared with 2-3, 100 µm polycarbonate

15   membranes and pre-equilibrated at 65°C. The suspension was extruded using ten passes at ~300 psi. After extrusion the formulation was processed using tangential flow diafiltration.

Tangential Flow Ultrafiltration. A 230 cm^2 Microcross tangential flow cartridge (50 kDa cut off) was attached to a Masterflex peristaltic pump, sample reservoir and buffer reservoir using Tygon tubing. The tubing length was adjusted so that the total circuit of

20   tubing, pump and TF cartridge had a total dead volume of 30 ml. To this system a 60 ml sample reservoir was attached. The sample was loaded into the tubing and reservoir by running the peristaltic pump at a low speed. After loading, the system was closed and the pump speed gradually increased to the pump maximum (approx. 100 ml/min) until the initial TF cartridge inlet pressure was 12-15 psi and the outlet pressure was 8-11 psi. When the system pressure

25   stabilized, both the filtrate outlet and the buffer reservoir were opened. Opening these valves allowed filtrate to flow out of the cartridge at ~ 10-15 ml/min while wash buffer (i.e. PBS, pH 7.5) was being collected. For a 50 - 60 ml formulation 700 - 900 ml of buffer was used to "wash" the sample. Fractions (10 ml) of the filtrate were collected for analysis of ethanol removal, pH, and antisense. After diafiltration was completed the wash buffer reservoir was closed and with

30   the pump continuing to run, filtrate was allowed to flow, concentrating the sample, typically reducing the preparation volume to the tubing dead volume (30 - 35 ml). The sample was

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collected from the system and the tubing and column were washed with 15 ml wash buffer to remove any remaining formulation.

Antisense Quantification. Antisense concentration was normally determined by measuring absorbance at 260nm as outlined in the current protocol. Briefly, antisense stock

5   solutions were quantified by diluting 1:500 in MilliQ water and measuring absorbance. TF filtrate fractions were diluted 1:10 in MilliQ water and absorbance was measured. Antisense in suspension with lipids was measured by adding 10 µl of the suspension to 250 µl MilliQ water. A monophase was created by adding 750 µl CHCl$_3$/MeOH (2.1:1) and 100 µl MeOH. Immediately after vortexing the mixture the absorbance was measured at 260 nm. In each case

10   the extinction coefficient for the given antisense was multiplied by the dilution factor to determine the antisense concentration.

Lipid Quantification. As outlined in the current protocol, 50 µl aliquots of the lipid/antisense suspension was diluted with 100 µl MilliQ water and submitted for analysis by HPLC. The percent **encapsulation efficiency** of the formulation is determined by dividing the

15   Drug/Lipid ratio of the finished product by the initial Drug/Lipid ratio formed when the lipid and antisense stock solutions are mixed.

Ethanol Assay. Ethanol in the TF filtrate was determined using an alcohol dehydrogenase reagent kit supplied by Sigma Chemical Co.

DEAE Sephadex chromatography. A suspension of the processed formulation

20   was loaded onto a 1 X 10 cm column of DEAE sephadex equilibrated in 20 mM PBS, pH 7.5. After eluting through the column the formulation was collected into a sterile falcon tube. The volume, antisense and lipid concentration were measured to determine recovery.

Particle Size. The particle size of the formulation was measured by QELS using a Nicomp Particle sizer, (Nicomp, Santa Barbara, CA.) and particle sizes are reported in the

25   particle mode with volume weighing.

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Results of Large Scale Preparations:

Assay	Initial Lipid Content (mg/ml)	Initial Antisense Content (mg/ml)	Final Lipid Content (mg/ml)	Final Antisense Content (mg/ml)	Initial Drug:Lipid	Final Drug:Lipid	Encaps. Effic.
A	10.581	1.936	14.604	1.681	0.183	0.115	63%
B	8.727	2.284	7.926	1.008	0.262	0.127	48%
C	11.06	2.97	2.69	0.556	0.286	0.207	77%

EXAMPLE 10

Phosphodiester and phosphorothioate antisense oligonucleotides encapsulated according to the methods in Example 2 and 5-9 were examined for their relative susceptibility to nuclease digestion by serum or S1 nuclease. Protection of the phosphodiester-linked oligonucleotide was significantly higher in serum when encapsulated as opposed to the free, raising the $T_{1/2}$ of degradation from 10 mins to at least 8h. Free phosphorothioate oligodeoxynucleotide showed significant breakdown in serum within 30 minutes, however encapsulated phosphorothioate oligodeoxynucleotide did not show any sign of degradation even after 24h incubation in serum. *In vivo* data agrees with these findings, showing no sign of degradation of the encapsulated phosphorothioate antisense until 8h.

As a positive control, the free phosphodiester and phosphorothioate antisense were subjected to very potent levels of S1 nuclease (100U/50μg) (1U of S1 nuclease will digest 1 ug DNA per minute at 37°C). The enzyme completely digested the free phosphodiester and phosphorothioate within seconds after its addition. The encapsulated phosphodiester under the same conditions was over 90% intact at 24h, and the encapsulated phosphorothioate was fully intact at 24h.

The experiments were conducted as described in the specification, or modified as follows.

S1 Nuclease Digestion. 50μg aliquots containing free, encapsulated, or encapsulated + 0.5% Triton X100 were aliquoted into 1.5 ml eppendorf tubes. To the tubes were added 10 μl 10X S1 nuclease buffer, dH2O (to make final volume 100 μl), and, just prior to digestion, 100U of S1 nuclease to each eppendorf tube. The tubes were sealed with parafilm and incubated at 55°C. A sample of the free, encapsulated, or encapsulated + 0.5% Triton X100 not digested by nuclease (standard) was frozen in liquid nitrogen in an eppendorf

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tube and stored at -20°C. At each desired time point, an aliquot of each sample was collected, added to GDP buffer containing proteinase K (133 μg/ml) and immediately frozen in liquid nitrogen in order to stop the reaction. Once all of the time points were collected, the samples were incubated at 55°C in a waterbath to activate proteinase K enabling it to

5   denature any remaining S1 nuclease. Proteinase K digested samples were applied to polyacrylamide gels, described below, to assess levels of S1 nuclease degradation

     Normal Murine/Human Serum Digestion. 50μg of the free, encapsulated, or encapsulated + 0.5% Triton X100 was aliquoted into 1.5 ml eppendorf tubes. To the tubes we added 45 μl normal murine/human serum, dH2O (to make final volume 50 μl), to each

10   eppendorf tube. The tubes were sealed with parafilm and incubated at 37°C. A sample of the free, encapsulated, or encapsulated + 0.5% Triton X100 not digested by nuclease (standard) was frozen in liquid nitrogen in an eppendorf tube and stored at -20°C. Aliquots were taken at various time points, added to GDP buffer containing proteinase K (133 μg/ml) and immediately frozen in liquid nitrogen to stop the reaction. Once all of the time points were

15   collected, the samples were incubated at 55°C in a waterbath to activate proteinase K enabling it to denature any remaining exonuclease. Proteinase K digested samples were applied to polyacrylamide gels to assess levels of exonuclease degradation

     Micrococcal Nuclease. An alternative standard nuclease assay not employed in the present experiment is the assay disclosed by Rahman et al. US Pat. 5665710, wherein nucleic acid/

20   lipid particles are incubated for 30 mins at 37°C in presence of an excess of micrococcal nuclease in 1 mM CaCl$_2$.

     Polyacrylamide Gel Electrophoresis (PAGE). Prepared 14 cm X 16 cm X 7.5mm polyacrylamide (15% or 20%) gels in 7M urea and TBE. Approximately 300 ng of sample (at each time point) and standard were aliquoted into eppendorf tubes. An equivalent

25   volume of 2X loading buffer was added to each sample. The samples were then heated in a waterbath to 90°C for 3 min to reduce secondary structures and then applied to the gel. The loaded gel was electrophoresed at 600V for 10 min (to sharpen the band) and then at 300V for the duration of the gel. The gel was incubated in 1X SyberGreen I stain in TBE for a minimum of 15 min and then photographed while illuminated under UV light (3.5 sec

30   exposure, 4.5 aperture).

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VII.   Conclusion

As discussed above, the present invention provides methods of preparing lipid-encapsulated therapeutic agent (nucleic acid) compositions in which the therapeutic agent (nucleic acid) portion is encapsulated in large unilamellar vesicles at a very high efficiency.

5   Additionally, the invention provides compositions prepared by the method, as well as methods of introducing therapeutic agents (nucleic acids) into cells. The compositions are surprisingly efficient in transfecting cells, both *in vivo* and *in vitro.*

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as

10   if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i) APPLICANT:
     (A) NAME: Inex Pharamceuticals Corporation
     (B) STREET: 100 - 8900 Glenlyon Parkway
     (C) CITY: Burnaby
     (D) PROVINCE: B.C.
     (E) COUNTRY: Canada
     (F) POSTAL CODE: V5J 5J8
     (G) TELEPHONE:
     (H) TELEFAX:
     (I) TELEX:

   (ii) TITLE OF INVENTION: High Efficiency Encapsulation of Charged
     Therapeutic Agents in Lipid Vesicles

   (iii) NUMBER OF SEQUENCES: 17

   (iv) CORRESPONDENCE ADDRESS:
     (A) ADDRESSEE: Smart & Biggar
     (B) STREET: Box 11560, Vancouver Centre
            2200 - 650 West Georgia Street
     (C) CITY: Vancouver
     (D) PROVINCE: B.C.
     (E) COUNTRY: Canada
     (F) POSTAL CODE: V6B 4N8

   (v) COMPUTER READABLE FORM:
     (A) MEDIUM TYPE: Floppy disk
     (B) COMPUTER: IBM PC compatible
     (C) OPERATING SYSTEM: PC-DOS/MS-DOS
     (D) SOFTWARE: Word Perfect

   (vi) CURRENT APPLICATION DATA:
     (A) APPLICATION NUMBER:
     (B) FILING DATE:
     (C) CLASSIFICATION:

   (vii) PRIOR APPLICATION DATA:
     (A) APPLICATION NUMBER: US 08/856,374
     (B) FILING DATE: 14-MAY-1997

   (viii) ATTORNEY/AGENT INFORMATION:
     (A) NAME: Smart & Biggar
     (C) REFERENCE/DOCKET NUMBER: 80472-2

   (ix) TELECOMMUNICATION INFORMATION:
     (A) TELEPHONE: (604) 682-7295
     (B) TELEFAX: (604) 682-0274

(2) INFORMATION FOR SEQ ID NO:1:

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```
                (i)  SEQUENCE CHARACTERISTICS:
                     (A)  LENGTH: 20
                     (B)  TYPE: nucleic acid
                     (C)  STRANDEDNESS: single
                     (D)  TOPOLOGY: linear

               (ii)  MOLECULE TYPE: other nucleic acid

              (iii)  HYPOTHETICAL: no

               (iv)  ANTI-SENSE: yes

               (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:1:

      TGCATCCCCC AGGCCACCAT                                               20


  (2)  INFORMATION FOR SEQ ID NO:2:

                (i)  SEQUENCE CHARACTERISTICS:
                     (A)  LENGTH: 20
                     (B)  TYPE: nucleic acid
                     (C)  STRANDEDNESS: single
                     (D)  TOPOLOGY: linear

               (ii)  MOLECULE TYPE: other nucleic acid

              (iii)  HYPOTHETICAL: no

               (iv)  ANTI-SENSE: yes

               (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:2:

       GCCCAAGCTG GCATCCGTCA                                              20


  (2)  INFORMATION FOR SEQ ID NO:3:

                (i)  SEQUENCE CHARACTERISTICS:
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                     (B)  TYPE: nucleic acid
                     (C)  STRANDEDNESS: single
                     (D)  TOPOLOGY: linear

               (ii)  MOLECULE TYPE: other nucleic acid

              (iii)  HYPOTHETICAL: no

               (iv)  ANTI-SENSE: yes

               (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:3:

       GGTGCTCACT GCGGC                                                   15
```

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(2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 15
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid

      (iii) HYPOTHETICAL: no

       (iv) ANTI-SENSE: yes

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AACGTTGAGG GGCAT   15

(2) INFORMATION FOR SEQ ID NO:5:

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             (A) LENGTH: 16
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid

      (iii) HYPOTHETICAL: no

       (iv) ANTI-SENSE: yes

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TAACGTTGAG GGGCAT   16

(2) INFORMATION FOR SEQ ID NO:6:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 24
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid

      (iii) HYPOTHETICAL: no

       (iv) ANTI-SENSE: yes

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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TATGCTGTGC CGGGGTCTTC GGGC   24

(2) INFORMATION FOR SEQ ID NO:7:

     (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

   (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTGCCGGGGT CTTCGGGC   18

(2) INFORMATION FOR SEQ ID NO:8:

     (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

   (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGACCCTCCT CCGGAGCC   18

(2) INFORMATION FOR SEQ ID NO:9:

     (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

   (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

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        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCCTCCGGAG CCAGACTT   18

(2) INFORMATION FOR SEQ ID NO:10:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 15
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid

        (iii) HYPOTHETICAL: no

        (iv) ANTI-SENSE: yes

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCGTGGTCAT GCTCC   15

(2) INFORMATION FOR SEQ ID NO:11:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 21
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid

        (iii) HYPOTHETICAL: no

        (iv) ANTI-SENSE: yes

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CAGCCTGGCT CACCGCCTTG G   21

(2) INFORMATION FOR SEQ ID NO:12:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 20
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid

        (iii) HYPOTHETICAL: no

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(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAGCCATGGT TCCCCCCAAC   20

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

    (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTTCTCGCTG GTGAGTTTCA   20

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

    (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TCTCCCAGCG TGCGCCAT   18

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

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```
     (iii) HYPOTHETICAL: no

      (iv) ANTI-SENSE: yes

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

   GTGCTCCATT GATGC                                                    15


(2) INFORMATION FOR SEQ ID NO:16:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 34
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid

      (iii) HYPOTHETICAL: no

       (iv) ANTI-SENSE: yes

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

   GAGUUGCUGA UGAGGCCGAA AGGCCGAAAG UCUG                              34


(2) INFORMATION FOR SEQ ID NO:17:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 15
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid

      (iii) HYPOTHETICAL: no

       (iv) ANTI-SENSE: yes

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

   GAACGGAGAC GGTTT                                                   15
```

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CLAIMS

1.   A method for preparation of a composition comprising lipid-encapsulated therapeutic agent particles, said method comprising the steps of:

5   (a)   combining a mixture of lipids comprising at least a first lipid component and a second lipid component with a buffered aqueous solution of a charged therapeutic agent to form an intermediate mixture containing lipid-encapsulated therapeutic agent particles, said first lipid component being selected from among lipids containing a protonatable or deprotonatable group that has a pKa such that the lipid is in a charged form at a first pH and a neutral form at a second pH, preferably near physiological pH, said buffered solution having a pH such that the first lipid component is in its charged form when in the buffered solution, said first lipid component being further selected such that the charged form is cationic when the charged therapeutic agent is anionic in the buffered solution, and anionic when the charged therapeutic agent is cationic in the buffered solution, and said second lipid component being selected from among lipids that prevent particle aggregation during lipid-therapeutic agent particle formation, and

            (b) changing the pH of the intermediate mixture to neutralize at least some exterior surface charges on said lipid-encapsulated therapeutic agent particles to provide at least partially-surface neutralized lipid-encapsulated therapeutic agent particles.

2.   The method of claim 1, wherein the therapeutic agent is a polyanionic nucleic acid.

3.   The method of claim 2, wherein said nucleic acid is an antisense nucleic acid.

4.   The method of claim 3, wherein said antisense nucleic acid contains linkages selected from the group consisting of phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate, phosphoroselenate and amidate linkages.

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5.   The method of any of claims 2 to 4, wherein said nucleic acid is an antisense nucleic acid.

6.   The method of any of claims 2 to 5, wherein said nucleic acid contains exclusively phosphodiester linkages.

7.   The method of claim 6, wherein the buffered solution comprises 10 to 50 mM citrate or phosphate buffer.

8.   The method of any of claims 2 to 5, wherein the nucleic acid contains at least some phosphorothioate or phosphordithioate linkages.

9.   The method of claim 8, wherein the buffered solution comprises 10 to 300 mM citrate or phosphate buffer.

10.   The method of claim 2, wherein said nucleic acid is a ribozyme.

11.   The method of any of claims 2 to 10, wherein said composition consists essentially of lipid-nucleic acid particles, said particles having a size of from 70 nm to about 200 nm.

12.   The method of any of claims 1 to 11, wherein said mixture of lipids in step (a) is a mixture of lipids in alcohol.

13.   The method of any of claims 1 to 12, wherein the first lipid component is an amino lipid.

14.   The method of any of claims 1 to 13, wherein the second lipid component is a polyethylene glycol-modified or polyamide oligomer-modified lipid.

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15.   The method of any of claims 1 to 14, wherein said lipids present in said lipid mixture comprises an amino lipid having a pKa of from about 5 to about 11, a neutral lipid, Chol and a PEG-modified or Polyamide oligomer-modified lipid.

16.   The method of claim 15, wherein said lipids are present in molar percents of about 25-45% neutral lipid, 35-55% Chol, 10-40% amino lipid and 0.5-15% PEG-modified or polyamide oligomer-modified lipid.

17.   The method of any of claims 1 to 16, wherein the second lipid component is a PEG-Ceramide.

18.   The method of claim 17, wherein said mixture of lipids comprises DODAP, DSPC, Chol and PEG-Cer14.

19.   The method of claim 18, wherein said lipids are present in molar percents of about 25-45% DSPC, 35-55% Chol, 10-40% DODAP and 0.5-15% PEG-Cer14.

20.   The method of claim 17, wherein said mixture of lipids comprises DODAP, POPC, Chol and PEG-Cer14.

21.   The method of claim 17, wherein said mixture of lipids comprises DODAP, SM, Chol and PEG-Cer14.

22.   The method of any of claims 1 to 21, wherein the step of changing the pH is performed using tangential flow dialysis.

23.   A composition comprising lipid-therapeutic agent particles comprising a lipid portion and a charged therapeutic agent, said charged therapeutic agent being encapsulated in said lipid portion, wherein said lipid portion comprises at least a first lipid component and a second lipid component, said first lipid component being selected from among lipids containing a protonatable or deprotonatable group that has a pKa such that the

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lipid is in a charged form at a first pH and a neutral form at a second PH, preferably near

physiological pH, and said first lipid component being further selected such that the charged

form is cationic when the therapeutic agent is anionic and anionic when the therapeutic agent

is cationic, and said second lipid component being selected from among lipids that prevent

5   particle aggregation during lipid-nucleic acid particle formation and which exchange out of

the lipid particle at a rate greater than PEG-CerC20.

        24.   A composition comprising lipid-therapeutic agent particles comprising

a lipid portion and a charged therapeutic agent, said charged therapeutic agent being

10   encapsulated in said lipid portion, wherein said lipid portion comprises at least a first lipid

component and a second lipid component, said first lipid component being selected from

among lipids containing a protonatable or deprotonatable group that has a pKa such that the

lipid is in a charged form at a first pH and a neutral form at a second pH, preferably near

physiological pH, and said first lipid component being further selected such that the charged

15   form is cationic when the therapeutic agent is anionic and anionic when the therapeutic agent

is cationic, and said second lipid component being selected from among lipids that prevent

particle aggregation during lipid-nucleic acid particle formation, said particles having a

nucleic acid/lipid ratio of at least 10% by weight and a size of from about 70 to about 200

nm.

20

        25.   The composition according to claim 23 or 24, wherein at least some of

the protonatable or deprotonatable groups disposed on the exterior surface of the particles

have been neutralized.

25   26.   The composition according to any of claims 23 to 25, wherein the

therapeutic agent is anionic.

        27.   The composition according to claim 26, wherein the therapeutic agent

is a polyanionic nucleic acid.

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28.   The composition according to claim 27, wherein the nucleic acid is an antisense nucleic acid.

29.   The composition according to claim 27 or 28, wherein at least 50% of the nucleic acid in the composition is encapsulated within the particle.

5

30.   The composition of claim 29, wherein at least 90% of the nucleic acid in the composition is encapsulated within the particle.

31.   The composition of any of claims 27 to 30, wherein the nucleic acid

10   has exclusively phosphodiester linkages.

32.   The composition of any of claims 27 to 31, wherein at least 50% of the nucleic acid in the composition is encapsulated within the particle.

15   33.   The composition of claim 32, wherein at least 90% of the nucleic acid in the composition is encapsulated within the particle.

34.   The composition of any of claims 27 to 30, wherein said nucleic acid is a ribozyme.

20

35.   The composition of any of claims 23 to 34, wherein the first lipid component is an amino lipid.

36.   The composition of any of claims 23 to 35, wherein the second lipid

25   component is a polyethylene glycol-modified or polyamide oligomer-modified lipid.

37.   The composition of any of claims 23 to 36, wherein the lipid portion comprises a neutral lipid, an amino lipid, cholesterol and PEG-modified or polyamide oligomer-modified lipid, and wherein said lipids are present at molar percents of about 25-

30   45% neutral lipid, 35-55% cholesterol, 10-40% amino lipid and 0.5-15% PEG-modified or polyamide oligomer-modified lipid.

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38.   The composition of claim 37, wherein said lipid portion comprises DODAP, DSPC, Chol and PEG-Cer14.

5

39.   The composition of claim 38, wherein the lipids are present in molar percents of about 25-45% DSPC, 35-55% Chol, 10-40% DODAP and 0.5-15% PEG-Cer14.

40.   The composition of claim 37, wherein said lipid portion comprises DODAP, POPC, Chol and PEG-Cer14.

10

41.   The composition of claim 37, wherein said lipid comprises of DODAP, SM, Chol and PEG-Cer14.

42.   A method for introducing a nucleic acid into a cell, comprising

15   contacting a cell with a lipid-nucleic acid composition prepared according to any of claims 1 to 22 for a period of time sufficient to introduce the nucleic acid into said cell.

43.   A method for the treatment or prevention of a disease characterized by aberrant expression of a gene in a mammalian subject comprising,

20   preparing a lipid-encapsulated therapeutic nucleic acid particle according to the method of any of claims 1 to 22, wherein the therapeutic nucleic acid component hybridizes specifically with the aberrantly expressed gene; and

administering a therapeutically effective or prophylactic amount of the particle to the mammalian subject, whereby expression of the aberrantly expressed gene is reduced. .

25

44.   The method of claim 43, wherein the gene is selected from among ICAM-1, c-myc, c-myb, ras, raf, erb-B-2, PKC-alpha, IGF-1R, EGFR, VEGF and VEGF-R-1.

45.   The method of claim 43, wherein the disease is a tumor.

30

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46.   The method of claim 43, wherein the disease is characterized by inflammation.

47.   The method of claim 43, wherein the disease is an infectious disease.

48.   The method of claim 43, wherein the therapeutically effective amount of the particle is administered to the mammalian subject by intravenous injection.

49.   The method of claim 48, wherein the therapeutically effective amount of the particle is administered to the mammalian subject by intravenous injection at an injection site, and wherein the disease is localized at a disease site distal to the injection site.

50.   The method of any of claims 43 to 49, wherein the particles are administered to the mammal in a plurality of doses, at intervals of from one to three weeks.

51.   The method of any of claims 43 to 50, wherein the nucleic acid comprises exclusively phosphodiester linkages.

52.   A method of preventing expression of a disease-associated gene in a mammalian cell comprising,

preparing a lipid-therapeutic oligonucleotide particle according to any of claims 1 to 22 containing an antisense therapeutic agent; and

exposing the mammalian cell to the lipid-therapeutic oligonucleotide particle for a period of time sufficient for the therapeutic oligonucleotide component to enter the cell;

wherein the antisense therapeutic agent has a sequence complementary to the disease-associated gene and reduces the production of the gene product of the disease-associated gene in the cell.

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53.   A pharmaceutical composition comprising lipid-therapeutic agent particles prepared according to any of claims 1 to 22 and a pharmaceutically acceptable carrier.

5

54.   A method for treatment or prevention of a disease characterized by aberrant expression of a gene in a mammalian subject comprising, administering to mammalian subject a composition comprising lipid-encapsulated nucleic acid particles, wherein the lipid- encapsulated nucleic acid particles contain at least 10% by weight of nucleic acids and the nucleic acids have exclusively phosphodiester linkages.

10

55.   A composition comprising lipid-encapsulated nucleic acid particles, wherein the lipid- encapsulated nucleic acid particles contain at least 10% by weight of nucleic acids and the nucleic acids have exclusively phosphodiester linkages.

15

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pH 4.0 INTERNAL

pH 4.0 EXTERNAL

COLUMN CHROMATOGRAPHY

1. EXCHANGE pH 4.0 CITRATE
    FOR pH 7.5 HBS

2. NEUTRALIZE SURFACE
    DODAP; ANTISENSE RELEASE

3. REMOVAL OF NON-
    ENCAPSULATED ANTISENSE

RELEASE OF
SURFACE
ANTISENSE

pH 4.0 INTERNAL

pH 7.5 EXTERNAL

FIG. 1

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DODAP: AL-1

OTHER AMINO LIPIDS:

R^1 AND/OR R^2 ARE H,
ALKYL OR FATTY ALKYL GROUPS
R^3 IS H, LOWER ALKYL.

R^{11} AND/OR R^{12} ARE LOWER ALKYL/LOWER ACYL, FATTY ALKYL,
FATTY ACYL.

(AT LEAST ONE OF R^{11} OR R^{12} IS A LONG CHAIN ALKYL OR ACYL
GROUP)

R^{13} AND R^{14} ARE EACH H, LOWER ALKYL.

FIG. 2A

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FIG. 2B

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FIG. 3

FIG. 4

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FIG. 5

FIG. 6

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IMMEDIATELY AFTER REMOVAL OF FREE ANTISENSE

VOLUME WEIGHTING:
MEAN DIAMETER = 119.3 nm
STD DEVIATION = 32.2 nm (27.0 %)
CUMULATIVE RESULTS:
25 % OF DISTRIBUTION <   88.60 nm
50 % OF DISTRIBUTION < 106.74 nm
75 % OF DISTRIBUTION < 127.93 nm
90 % OF DISTRIBUTION < 151.04 nm
99 % OF DISTRIBUTION < 199.22 nm

AFTER 2 MONTH STORAGE AT 4°C

VOLUME WEIGHTING:
MEAN DIAMETER = 114.2 nm
STD DEVIATION = 27.8 nm (24.3%)
CUMULATIVE RESULTS:
25 % OF DISTRIBUTION <   86.96 nm
50 % OF DISTRIBUTION < 102.86 nm
75 % OF DISTRIBUTION < 121.31 nm
90 % OF DISTRIBUTION < 140.78 nm
99 % OF DISTRIBUTION < 183.74 nm

FIG. 7

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FIG. 8

FIG. 9

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FIG. 10

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FIG. 11

FIG. 12

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FIG. 13

FIG. 14

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FIG. 15

FIG. 16

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FIG. 17

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FORMULATION #

- ● 1
- ■ 5 (NO AS)
- ▲ 2
- ▼ 4 (NO AS)
- ◆ 4
- ● 6
- ● 7

FIG. 18
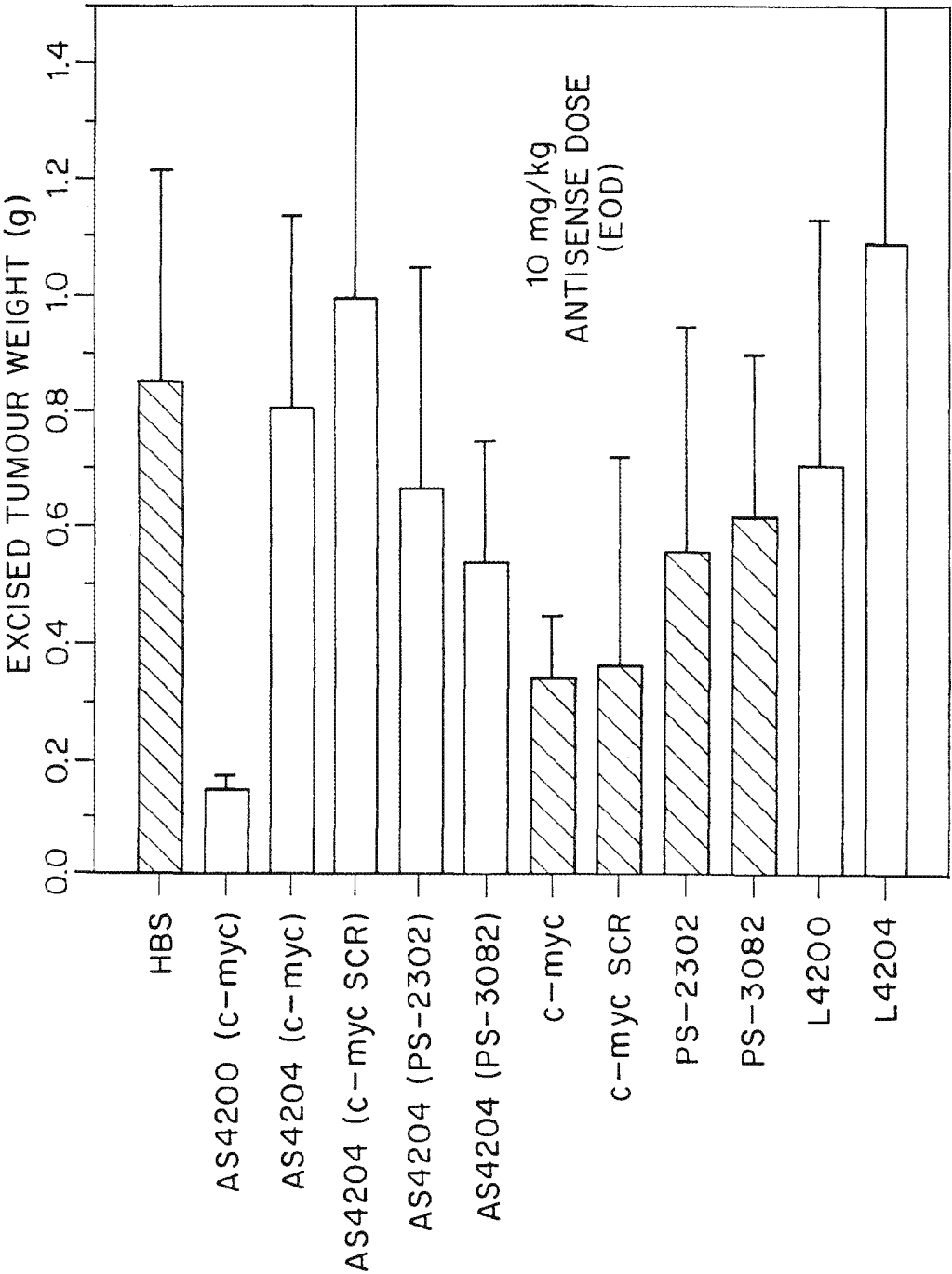
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GENV-00011215

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FIG. 19

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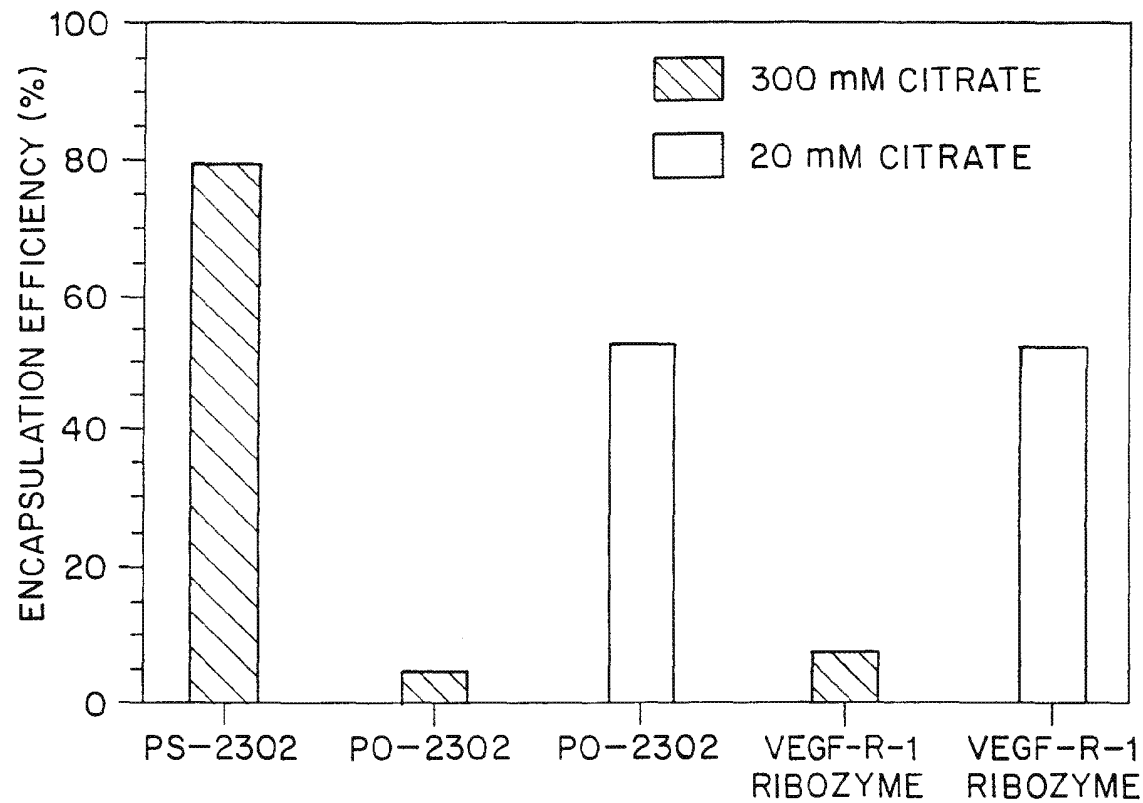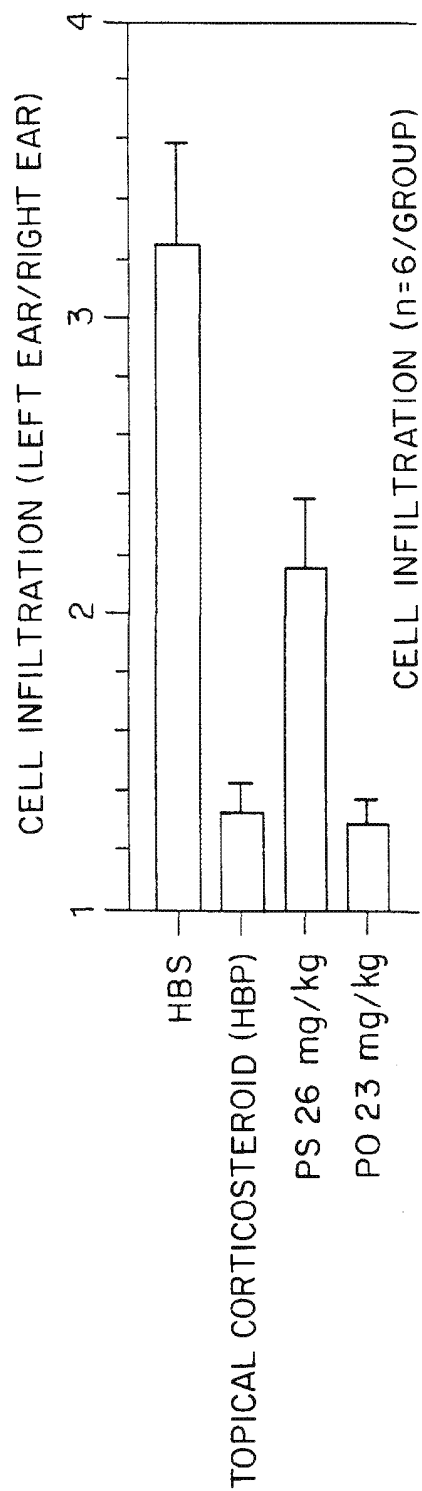

FIG. 20

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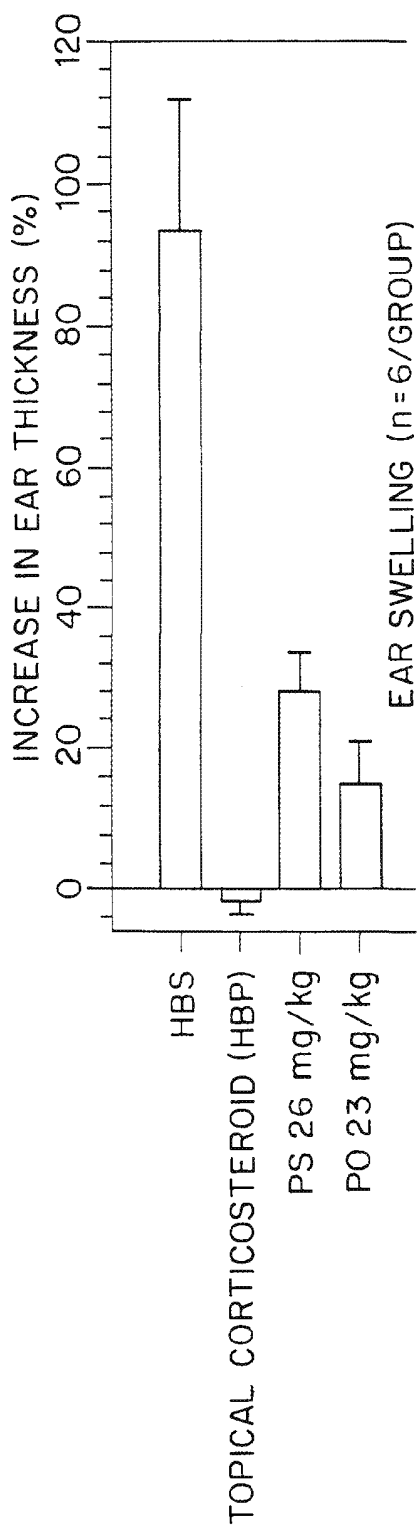
FIG. 21

FIG. 22

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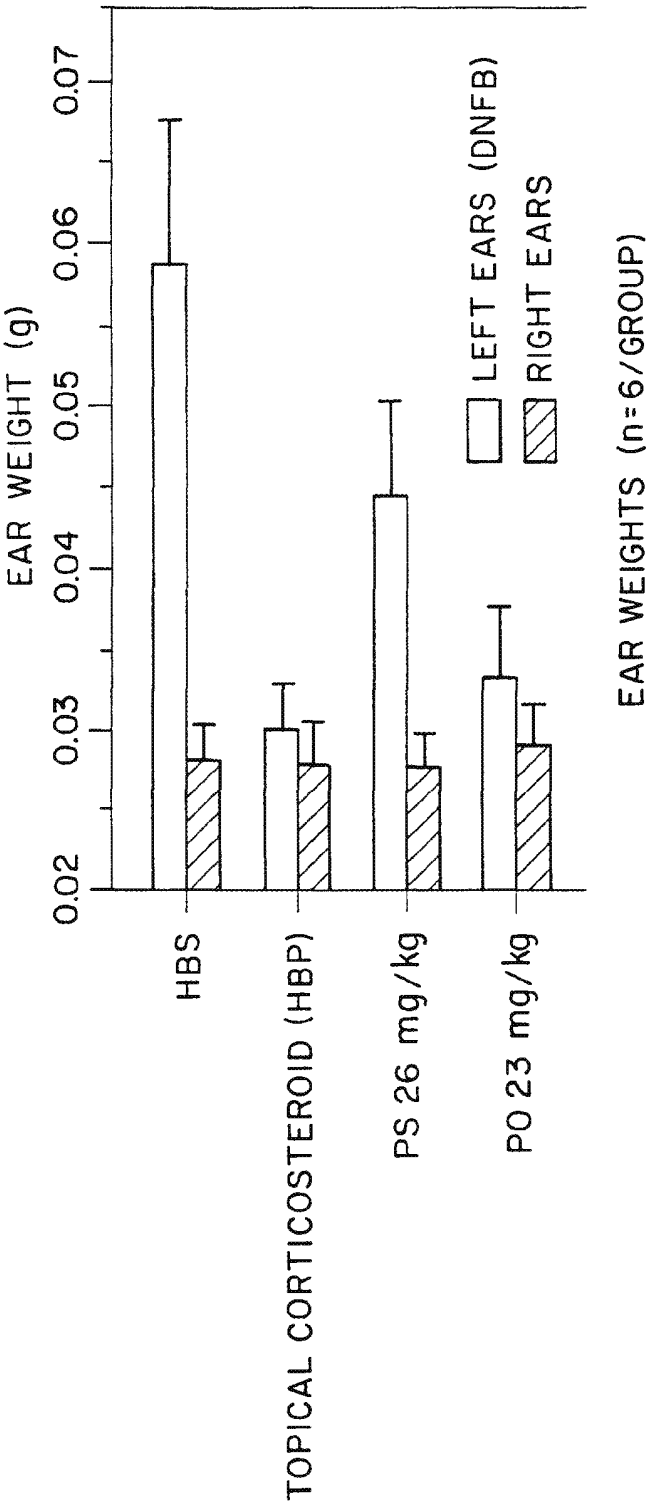17/17

FIG. 23

JOINT APPENDIX 42

ACQUIRED DISEASES   RESEARCH ARTICLE

Stabilized plasmid-lipid particles for systemic gene therapy

P Tam[1]*, M Monck[1]*, D Lee[1], O Ludkovski[1], EC Leng[1], K Clow[1], H Stark[2], P Scherrer[3], RW Graham[1] and PR Cullis[1,3]

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*The structure of 'stabilized plasmid-lipid particles' (SPLP) and their properties as systemic gene therapy vectors has been investigated. We show that SPLP can be visualized employing cryo-electron microscopy to be homogeneous particles of diameter 72 ± 5 nm consisting of a lipid bilayer surrounding a core of plasmid DNA. It is also shown that SPLP exhibit long circulation lifetimes (circulation half-life >6 h) following intravenous (i.v.) injection in a murine tumor model resulting in accumulation of up to 3% of the total injected dose and concomitant reporter gene expression at a distal (hind flank) tumor site. In contrast, i.v. injection of naked plasmid DNA or plasmid DNA–cationic liposome complexes did not result in significant plasmid delivery to the tumor site or gene expression at that site. Furthermore, it is shown that high doses of SPLP corresponding to 175 μg plasmid per mouse are nontoxic as assayed by monitoring serum enzyme levels, whereas i.v. injection of complexes give rise to significant toxicity at dose levels above 20 μg plasmid per mouse. It is concluded that SPLP exhibit properties consistent with potential utility as a nontoxic systemic gene therapy vector. Gene Therapy (2000) **7**, 1867–1874.*

Keywords: *liposomes; cancer gene therapy; intravenous gene therapy; tumour transfection*

Introduction

Gene therapies for systemic diseases such as cancer or inflammatory disorders clearly require systemic vectors. However, currently available vectors for gene therapy have limited utility for systemic applications. Recombinant virus vectors, for example, are rapidly cleared from the circulation following intravenous injection, limiting potential transfection sites to 'first pass' organs such as the liver.[1,2] Nonviral systems, such as plasmid DNA–cationic liposome complexes, are also rapidly cleared from the circulation, and the highest expression levels are again observed in first pass organs, particularly the lungs.[3–8]

Intravenous administration of chemotherapeutic drugs encapsulated in small (diameter ⩽100 nm), long-circulating (circulation half-life $t_{\frac{1}{2}}$ ⩾5 h in murine models) liposomes results in preferential delivery of encapsulated drug to distal tumors due to increased vascular permeability in these regions.[9–11] It therefore follows that intravenous injection of plasmid DNA encapsulated in small, long circulating lipid particles should give rise to preferential delivery of plasmid DNA to tumor sites. Recent work has shown that plasmid DNA can be encapsulated in small (approximately 70 nm diameter) 'stabilized plasmid-lipid particles' (SPLP) that contain one plasmid per particle.[12] These particles contain the 'fusogenic'

lipid dioleoylphosphatidylethanolamine (DOPE), low levels of cationic lipid and are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. Here, we show that the structure of SPLP can be directly visualized employing cryo-electron microscopy to reveal homogeneous particles consisting of plasmid DNA entrapped within a bilayer lipid vesicle. Furthermore, we show that these SPLP exhibit long circulation lifetimes and no evidence of systemic toxicities following i.v. injection in a murine tumor model. Under the experimental conditions employed, approximately 3% of the total injected SPLP dose was delivered to a subcutaneous tumor site and 1.5 % of the total intact plasmid dose could be detected at the tumor site at 24 h. Significant levels of reporter gene expression were observed at the tumor site employing the SPLP system, whereas no expression was observed following i.v. injection of 'naked' plasmid DNA or plasmid DNA–cationic liposome complexes.

Results

SPLP consist of a plasmid trapped inside a bilayer lipid vesicle

Previous work has shown that plasmid DNA can be encapsulated (trapping efficiency approximately 70%) in SPLP by a detergent dialysis procedure employing octylglucopyranoside (OGP).[12] These SPLP are composed of DOPE, 5–10 mol% of the cationic lipid dioleoyldimethylammonium chloride (DODAC) and PEG attached to a ceramide anchor containing an arachidoyl acyl group

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(PEG-CerC$_{20}$). SPLP can be separated from non-encapsulated plasmid by ion exchange chromatography and can then be further purified by density gradient centrifugation to remove empty vesicles produced during the dialysis procedure. On the basis of the size and plasmid-to-lipid ratio of these purified SPLP it was determined that each SPLP contained one plasmid molecule.[12]

Here, we further characterize SPLP structure employing cryo-electron microscopy. Following the procedures summarized in Materials and methods, purified SPLP were prepared from DOPE:DODAC:PEG-CerC$_{20}$ (83:7:10; mol:mol:mol) and pCMVluc, whereas large unilamellar vesicles (LUV) with the same lipid composition were prepared by extrusion of the hydrated lipid mixture through 100 nm pore size filters. As shown in Figure 1a, the cryo-electron micrographs clearly reveal SPLP to con-

sist of a lipid bilayer surrounding an internal structure consistent with entrapped plasmid DNA molecules. Small (diameter approximately 30 nm), empty vesicles formed during the detergent dialysis process[13] that were not removed by density centrifugation do not exhibit such internal structure (see arrows in Figure 1a). This internal structure is also not observed in the LUV produced by extrusion (Figure 1b). It may also be noted that SPLP as detected by cryo-electron microscopy have a remarkably homogeneous size (diameter 72 ± 5 nm), in close agreement with measurements of SPLP diameter employing freeze–fracture electron microscopy (diameter 64 ± 9 nm).[12] The homogeneous size and morphology of SPLP contrasts with the irregular morphology and large size distribution of the extruded vesicles. The narrow size distribution of SPLP was also reflected by quasi-elastic light scattering (QELS) measurements (data not shown) which indicated a mean diameter of 83 ± 4 nm. Plasmid DNA–cationic liposome complexes made from DOPE:DODAC (1:1; mol:mol) LUV exhibited a large, heterogeneous size distribution as determined by QELS (diameter 220 ± 85 nm, data not shown).

SPLP exhibit extended circulation lifetimes, preferential accumulation at tumor sites, and low systemic toxicities following intravenous injection

The next set of experiments was aimed at characterizing the pharmacokinetics and biodistribution of SPLP following i.v. injection into tumor-bearing mice. SPLP were prepared with trace amounts of the lipid label, ^{3}H-cholesteryl hexadecylether (^{3}H-CHE) and were injected at a dose level equivalent to 100 μg plasmid DNA per mouse into C57Bl/6 mice bearing a subcutaneous Lewis lung carcinoma (approximately 200 mg) in the hind flank. The clearance of SPLP from the circulation as assayed by the lipid label (Figure 2a) corresponds to a first order process with a $t_{\frac{1}{2}}$ of 6.4 ± 1.1 h. Relatively low levels of uptake by the lung and liver are observed (Figure 2b and c) whereas approximately 3% of the injected SPLP dose accumulates at the tumor site over 24 h (Figure 2d). Such tumor accumulation levels are comparable with those achieved for small, long-circulating liposomes containing conventional drugs such as doxorubicin, where approximately 5% of the injected dose can be found at 24 h in larger (>0.5 g) tumors.[14] In contrast to the behavior of the SPLP system, ^{3}H-CHE-labeled plasmid DNA–cationic liposome complexes were rapidly cleared from the circulation ($t_{\frac{1}{2}} \ll 15$ min), appearing predominantly in the lung and liver, and less than 0.2% of the injected dose was found at the tumor site at 24 h. The biodistribution of ^{3}H-CHE labeled SPLP and complexes 4 and 24 h following injection are summarized in Table 1. Only trace amounts were detected in kidney, heart and lymph nodes.

The levels of intact plasmid DNA in the circulation and tumor tissue following i.v. injection of naked plasmid DNA, plasmid DNA–cationic lipid complexes and SPLP were analyzed by Southern blot hybridization (Figure 3a, b and c, respectively) and quantified by phosphor-imaging analysis (Figure 3d and e). For naked plasmid, less than 0.01% of the injected dose remained intact in the circulation at 15 min, and no intact tumor-associated plasmid could be observed at any time. For plasmid administered in complexes, only a small fraction (<2%) was still intact in the circulation at 15 min and less than 0.2% was found to be intact in tumor tissue at 1 h. In
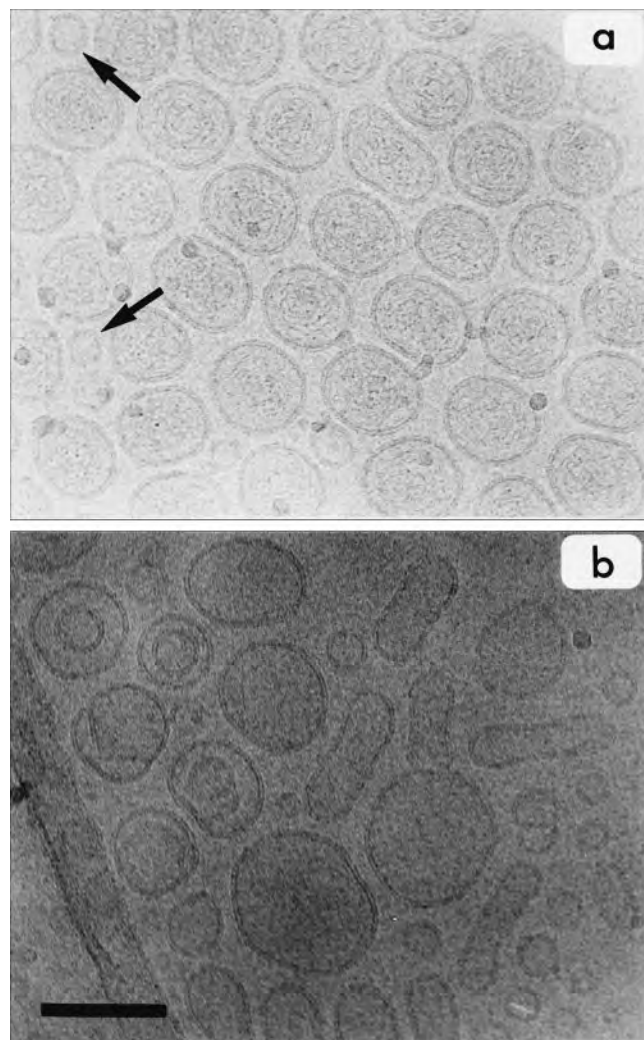

Figure 1 Cryo-electron micrographs of (a) purified SPLP and (b) LUV prepared by extrusion. SPLP were prepared from DOPE:DODAC:PEG-CerC$_{20}$ (83:7:10; mol:mol:mol) and pCMVluc and purified employing DEAE column chromatography and density gradient centrifugation. LUV were prepared from DOPE:DODAC:PEG-CerC$_{20}$ (83:7:10; mol:mol:mol) by hydration and extrusion through filters with 100 nm diameter pore size. The arrows in panel (a) indicate the presence of residual 'empty' vesicles formed during the detergent dialysis process that were not removed by the density centrifugation purification step. The bar in panel (b) indicates 100 nm. For details of sample preparation and cryo-electron microscopy see Materials and methods.
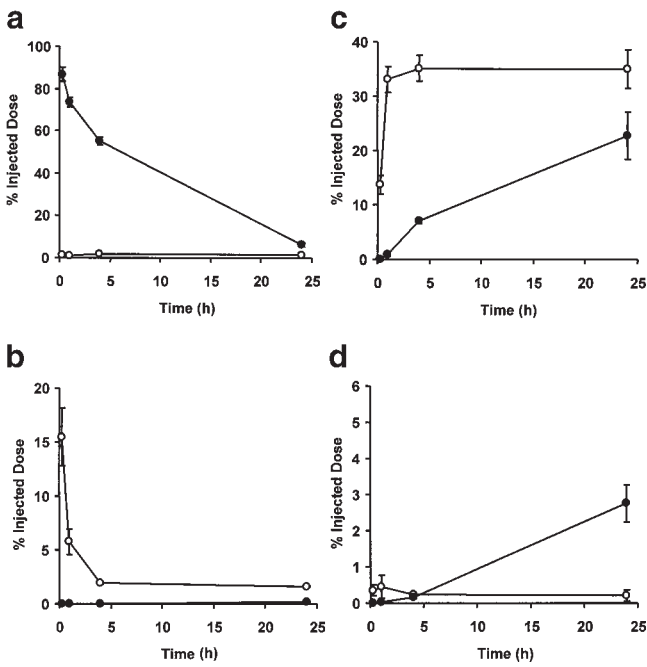
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Figure 2 Pharmacokinetics, tissue distribution and tumor accumulation of SPLP and plasmid DNA–cationic liposome complexes following intravenous administration in tumor-bearing mice as reported by the ^{3}H-CHE lipid marker. The levels of complexes (○) and SPLP (●) in the circulation, the lung, the liver and in Lewis lung tumor tissue are shown in panels (a), (b), (c) and (d), respectively. The accumulations in liver, lung and tumor were corrected for plasma contributions[29] and are expressed as a percentage of the total injected dose.

Table 1 Biodistribution of SPLP and plasmid DNA–cationic liposome complexes in mice 4 and 24 h following i.v. injection

Tissue	% Injected dose (s.e.m.)			
	SPLP		Complexes	
	4 h	24 h	4 h	24 h
Plasma	55.0 (1.7)	6.4 (1.0)	1.7 (0.2)	1.4 (0.3)
Liver	7.0 (0.6)	23.0 (4.3)	35.2 (2.3)	35.1 (3.5)
Lung	0.0 (0.1)	0.2 (0.1)	1.8 (0.8)	0.5 (0.0)
Spleen	0.4 (0.1)	1.6 (0.1)	0.2 (0.2)	0.1 (0.3)
Tumor	0.2 (0.0)	2.8 (0.5)	0.2 (0.1)	0.3 (0.2)

Both SPLP and complexes contained pCMVLuc as well as trace levels of the ^{14}C-labeled CHE lipid marker and were administered at a dose level of 100 μg plasmid per mouse. The biodistribution was measured employing the CHE lipid marker.
s.e.m., standard error of the mean.

contrast, following i.v. injection of SPLP, approximately 85% of the injected plasmid DNA remained in intact form in the circulation at 15 min, and progressively higher levels of intact plasmid accumulated at the tumor site over the time-course of the experiment. The levels achieved at 24 h correspond to approximately 1.5% of the total injected plasmid DNA dose. The circulation half-life of intact plasmid DNA following injection of SPLP was calculated to be 7.2 ± 1.6 h, in good agreement with the circulation half-life of ^{3}H-CHE-labeled SPLP, confirming the highly stable nature of SPLP in the circulation.

Serum enzyme levels of alanine aminotransferase

(ALT) or aspartate aminotransferase (AST) were assayed for evidence of toxicity following i.v. administration of SPLP and plasmid DNA–cationic liposome complexes. Elevated ALT and AST levels are usually associated with liver damage, although elevated AST levels can also indicate systemic tissue damage. Mice receiving SPLP at dose levels as high as 175 μg plasmid DNA per mouse did not have significantly elevated serum levels of ALT and AST (Figure 4a). However, mice receiving doses of plasmid DNA–cationic liposome complexes corresponding to plasmid doses above 20 μg per mouse exhibited progressively higher serum levels of ALT and AST, reaching levels 100-fold above normal levels at plasmid doses of 75 μg (Figure 4b).

Intravenously administered SPLP promote gene expression in a distal tumor

It is of obvious interest to determine whether SPLP-mediated delivery of intact plasmid to the tumor site results in transgene expression at that site. Luciferase gene expression in tumor tissue was therefore monitored following i.v. injection of SPLP, naked plasmid DNA and plasmid DNA–cationic liposome complexes at dose levels corresponding to 100 μg plasmid DNA per mouse. This dose level corresponded to the maximum tolerated dose of complexes as evidenced by animal morbidity and mortality. As shown in Figure 5, administration of SPLP results in reporter gene expression at the tumor site, with maximum levels corresponding to 32 pg luciferase per gram of tumor tissue at the 48 h time-point and significant gene expression extending to 96 h after injection. Injection of free plasmid DNA or plasmid DNA–cationic liposome complexes, on the other hand, resulted in no detectable gene expression at the tumor site. It is of interest to note that i.v. administration of complexes did result in transfection in the lung, liver and spleen, whereas administration of SPLP did not result in detectable levels of gene expression in these organs (data not shown). In an attempt to understand why SPLP did not give rise to significant gene expression in the liver, the levels of intact plasmid in the liver 24 h after injection of SPLP into C57Bl/6 mice (100 μg plasmid per mouse) bearing a subcutaneous Lewis lung carcinoma were analyzed by Southern blot hybridization. No intact plasmid could be detected in the liver whereas intact plasmid was readily detected at the tumor site (results not shown). This suggests that the ability of SPLP to transfect cells at the tumor site but not in the liver may reflect relatively rapid breakdown of SPLP and associated plasmid following uptake into liver phagocytes (Kuppfer cells), which play a dominant role in clearing liposomal systems from the circulation.[15] Lower gene expression in the liver may also reflect the finding that nonviral vectors such as SPLP transfect dividing cells much more efficiently than non-dividing cells[16] or that Kuppfer cells are less readily transfected than tumor cells.

Discussion

This study demonstrates that SPLP consist of plasmid DNA encapsulated in a bilayer vesicle, and that systemic administration of SPLP results in significant accumulation and transfection at a distal tumor site. There are three important features of these results. The first concerns the structure of SPLP, which represents a major
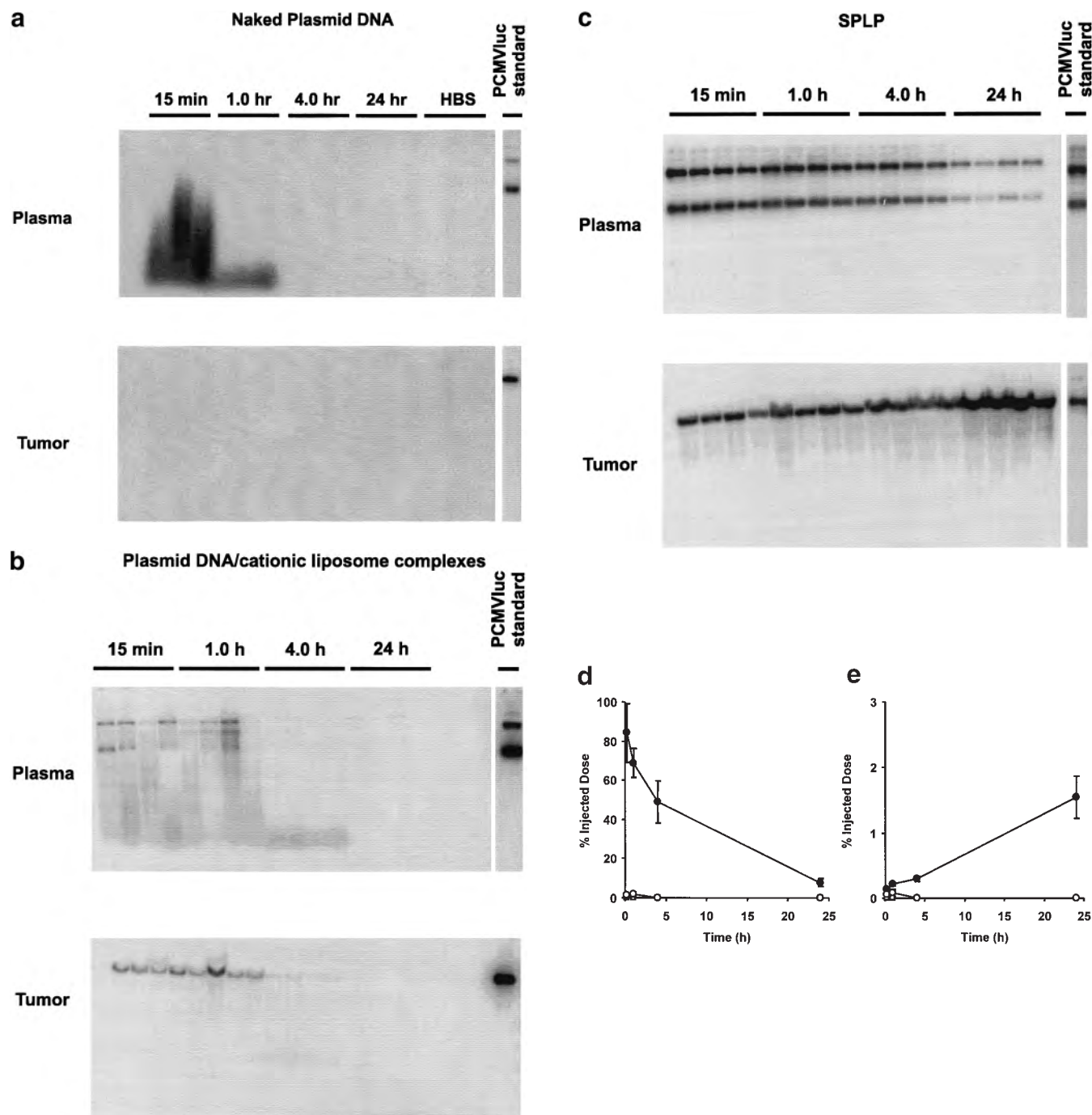
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Figure 3 Pharmacokinetics and tumor accumulation of plasmid DNA following intravenous administration of naked plasmid, plasmid DNA–cationic liposome complexes and SPLP as reported by a Southern blot analysis. The Southern blot hybridizations shown in panels (a), (b) and (c) result from plasmid DNA isolated from blood and tumor tissue of mice injected with naked plasmid DNA, plasmid DNA–cationic lipid complexes and SPLP, respectively. Each panel shows pCMVluc (2 ng) to indicate the position of intact plasmid DNA. The levels of intact plasmid resulting from i.v. injection of naked plasmid DNA (□), plasmid DNA–cationic liposome complexes (○) and SPLP (●) were quantified for plasma (panel d) and tumor tissue (panel e) by phospor-imaging analysis and converted to mass quantities of plasmid DNA by comparison to a standard curve made from known amounts of plasmid DNA. Tumor accumulations of plasmid were corrected for plasma contributions and expressed as a percentage of the total injected plasmid DNA dose.[29]

advance for plasmid encapsulation in liposomal delivery systems. Second, it is of interest to compare the properties of the SPLP system for systemic gene delivery and distal tumor transfection with the properties of other viral or nonviral gene delivery systems. Finally, the possi-

bilities for further optimization of the SPLP system are of interest. We discuss these areas in turn.

The cryo-electron microscopy results presented here establish the structure of SPLP as a plasmid surrounded by a lipid bilayer envelope. This represents the first direct
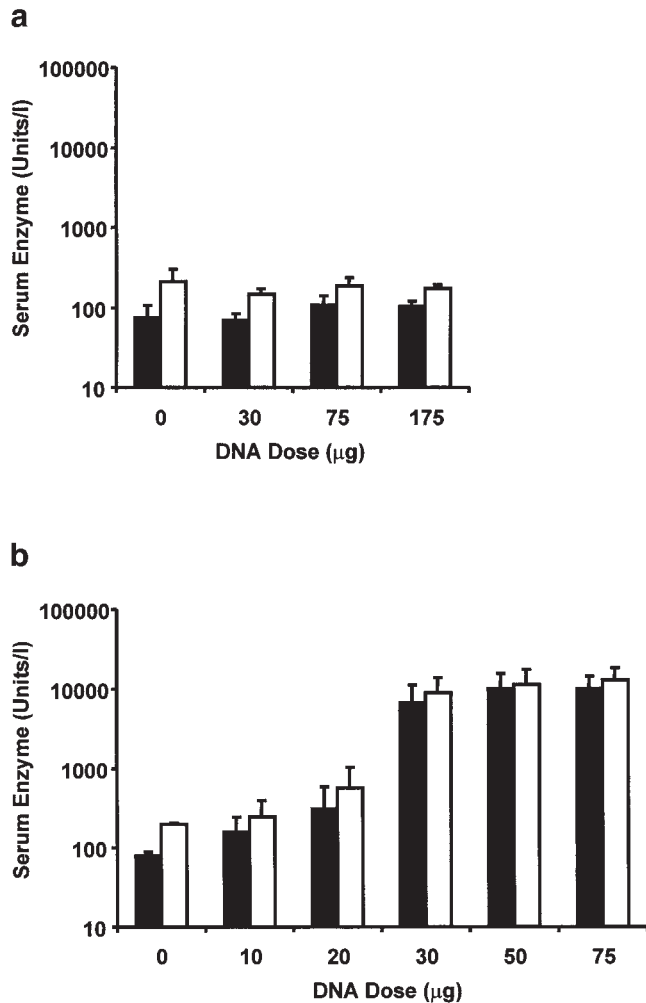
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a

b

Figure 4 Toxicity resulting from i.v. injection into mice of varying amounts of SPLP (panel a) and plasmid DNA–cationic lipid complexes (panel b) as assayed by determining serum levels of the hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (ASP). The serum levels of AST (□) and ALT (■) were measured 24 h after injection.
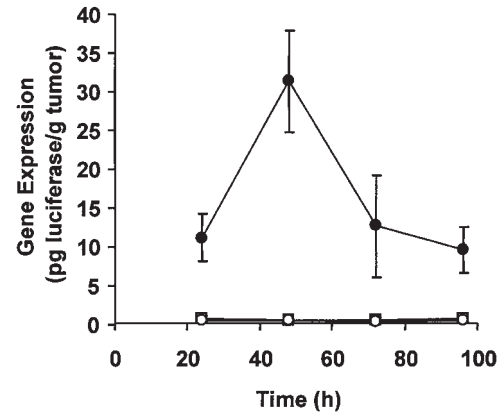

Figure 5 Transgene expression at a distal tumor site following intravenous injection of naked plasmid DNA (□), plasmid DNA–cationic lipid complexes (○) and SPLP (●). Mice bearing subcutaneous Lewis lung tumors were injected i.v. with doses containing 100 μg of pCMVluc. Tumors were harvested at the indicated times and assayed for luciferase activity. The level of transgene expression reported is normalized for the weight of the tumor tissue.

demonstration that plasmid can be entrapped in small (diameter approximately 70 nm), well defined vesicular systems containing a single plasmid per vesicle. Entrapment of a plasmid such as pCMVluc, which contains 5650 bp, in a supercoiled configuration in a 70 nm diameter vesicle represents a solution for a difficult packing problem. For example, electron micrographs of supercoiled 4.4 kbp plasmids reveals extended lengths of approximately 500 nm and average (two dimensional) diameters in the range of 350 nm, suggesting an average diameter for free supercoiled pCMVluc of approximately 400 nm.[17] The detergent dialysis process clearly involves a partial condensation of entrapped plasmid to allow encapsulation in a 70 nm diameter vesicle. The mechanism of entrapment is not understood in detail, but appears to proceed via association of plasmid with lipid structures formed as intermediates in the detergent dialysis process.[12]

SPLP exhibit extended circulation lifetimes ($t_\frac{1}{2}$ approximately 7 h) following i.v. injection, can deliver significant amounts of intact plasmid to a distal tumor site and

enable transgene expression corresponding to 30 pg luciferase per gram tumor at that site with no evidence of toxicity (as indicated by serum enzyme levels). Delivery of approximately 3% of the injected dose of SPLP at a 200 mg tumor site corresponds to more than 1000 plasmid copies per tumor cell, assuming a cell density of 1×10^9 per milliliter. It is of interest to compare these properties with the behavior of other gene delivery systems. In the case of viral vectors, there have been three reports of transgene expression in liver metastases and in a distal tumor following systemic administration of a recombinant vaccinia virus[18,19] and a selectively replicating adenovirus.[20] These viral vectors are replication incompetent in normal nondividing cells but can selectively replicate in tumor cells resulting in transgene expression in tumors and antitumoral efficacy. The major drawback of these viral vectors is the immune response, which occurs within 6 days. In the case of nonviral vectors such as plasmid DNA–cationic polymer 'polyplexes', there is only one report showing transfection of distal tumors following i.v. injection.[21] This work utilized a PEG-containing polyplex that exhibits plasmid circulation half-lives of less than 0.5 h following intravenous injection and gave rise to transfection at a distal tumor site, achieving transfection levels corresponding to approximately 250 pg/g tumor, approximately eight-fold higher than the levels reported here.

With regard to plasmid DNA–cationic liposome complexes ('lipoplexes'), a number of studies have characterized transfection properties following i.v. administration,[3–7] however, only two studies by Xu and co-workers have demonstrated transfection at a distal tumor site.[22,23] In the initial study,[22] less than 5% of the cells at the tumor site were transfected as indicated by immunohistochemical staining, whereas in the second study using transferrin targeted complexes 20–30% of the cells were transfected. The levels of gene expression could not be related to the levels observed here. Issues related to circulation lifetimes, plasmid tumor accumulation and toxicity were not addressed. An additional study[24] has demonstrated the presence of complexes at a distal tumor site following i.v. injection but the levels of

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gene transfer were not measured. In general, i.v. injection of complexes gives rise to high levels of transgene expression in the lungs, with lower levels of expression in the spleen, liver, heart and kidneys. Similar results were observed for the complexes employed in this investigation. The lung expression appears to arise from deposition in lung microvasculature and reflects the rapid clearance of plasmid DNA–cationic lipid complexes from the circulation due to their large size (>200 nm diameter) and high cationic lipid content.[8] This is consistent with the observation that murine B16 tumors seeded in the pulmonary vascular compartment can be transfected by i.v. administered complexes.[3] Finally, as clearly shown in this study, administration of complexes is often associated with significant toxicity.

The final point of discussion concerns the utility of SPLP as a systemic gene therapy vector and the potential for further optimization. As indicated above, despite the delivery of large amounts of intact plasmid to the tumor site, the levels of gene expression observed for the SPLP system are modest, albeit comparable with or superior than can be achieved with other vectors. It is likely that the low levels of transfection reflect low levels of uptake of SPLP into cells at the tumor site due to inhibition of cell association and uptake by the PEG coating.[25] *In vitro* studies have shown that SPLP containing PEG-CerC$_{20}$ are accumulated into cells to a very limited extent, however, the SPLP that are taken up are highly transfection potent.[26] The challenge that faces the next stage of SPLP development is, therefore, to devise methods of enhancing intracellular delivery of SPLP following arrival at the tumor site. There are a number of avenues to explore. First, the dissociation rate of the PEG coating from the SPLP can be modulated by varying the acyl chain length of the ceramide anchor,[12] suggesting the possibility of developing PEG-Cer molecules that remain associated with the SPLP long enough to promote passive targeting to the tumor, but which dissociate quickly enough to allow transfection after arriving at the tumor site. Alternatively, improvements may be expected from inclusion of cell-specific targeting ligands in SPLP to promote cell association and uptake. Finally, the nontoxic properties of SPLP allow the possibility of higher doses. A dose of 100 μg plasmid DNA per mouse corresponds to a dose of approximately 5 mg plasmid DNA per kilogram body weight. This is a relatively low dose level in comparison to small molecules used for cancer therapy, which typically are used at dose levels of 10 to 50 mg per kg body weight.

In summary, we have shown that SPLP consist of plasmid encapsulated in a lipid vesicle. Furthermore, we have demonstrated that, in contrast to naked plasmid or complexes, SPLP exhibit extended circulation lifetimes following intravenous injection, resulting in plasmid accumulation and transgene expression at a distal tumor site in a murine model. The levels of transgene expression achieved are modest, but are comparable or superior to distal tumor expression levels achieved employing other vectors. Further improvements can be expected due to the low toxicity and flexible nature of the SPLP system.

Materials and methods

Lipids and plasmid
1,2-Dioleoyl-3-phosphatidylethanolamine (DOPE) was obtained from Northern Lipids (Vancouver, BC, Canada).

The cationic lipid N,N-dioleyl-N,N-dimethylammonium chloride (DODAC) and 1-O-[2′-(ω-methoxypolyethylene-glycol)succinoyl]-2-N-arachidoylsphingosine (PEG-CerC$_{20}$) were prepared at Inex Pharmaceuticals (Burnaby, BC, Canada) using previously described methods.[27] ^{3}H-labeled cholesteryl hexadecyl ether (CHE) was purchased from Dupont NEN Products (Boston, MA, USA). The pCMVluc plasmid encodes the *Photinus pyralis* luciferase gene under the control of the human CMV immediate–early promoter. Plasmid DNA was propagated in *E. coli* (DH5α) and purified using the alkaline lysis method followed by two rounds of CsCl/ethidium bromide density equilibrium centrifugation.

SPLP and plasmid DNA–cationic liposome complex preparation
Plasmid DNA was encapsulated in SPLP composed of DOPE/DODAC/PEG-CerC$_{20}$ (83:7:10; mol:mol:mol) by the detergent dialysis method.[12] Lipids were dissolved in ethanol and dried to a lipid film in a round-bottom flask. The lipid mixture was resuspended in HBS (5 mM Hepes, 150 mM NaCl, pH 7.5) containing 200 mM OGP and 0.4 mg/ml pCMVluc. The final lipid concentration was 10 mg/ml. When required, ^{3}H-CHE was added to a specific activity of 1.0 μCi/mg total lipid. The mixture of lipid, plasmid and OGP was dialyzed against 4 l of HBS for 2 days with three changes. Untrapped plasmid was removed by DEAE-Sepharose CL-6B chromatography, and plasmid DNA-containing SPLP were purified by sucrose gradient centrifugation (2.5%/5%/10%) in a Beckman SW 28 rotor (16 h at 107 000 *g*) (Beckman, Fullerton, CA, USA). DNA-containing particles banding at the 5%/10% sucrose interface were collected and concentrated by ultrafiltration before the DNA concentration was adjusted to 500 μg/ml. The final lipid composition was determined by HPLC analysis. DNA was quantified by picogreen (Molecular Probes, Eugene, OR, USA) fluorescence of TX-100-solubilized SPLP preparations. Plasmid DNA–cationic liposome complexes were prepared by adding pCMVluc to large unilamellar vesicles (LUV) composed of DOPE:DODAC (1:1; mol:mol) to a final charge ratio (+/−) of 3.0 in 5% glucose. The LUV were prepared by extrusion through 100 nm pore size filters according to standard procedures.[28]

Cryo-electron microscopy
A drop of buffer containing SPLP was applied to a standard electron microscopy grid with a perforated carbon film. Excess liquid was removed by blotting leaving a thin layer of water covering the holes of the carbon film. The grid was rapidly frozen in liquid ethane, resulting in vesicles embedded in a thin film of amorphous ice. Images of the vesicles in ice were obtained under cryogenic conditions at a magnification of 66 000 and a defocus of −1.5 micron using a Gatan cryo-holder in a Philips CM200 FEG electron microscope (Eindhoven, The Netherlands).

Quasi-elastic light scattering
The mean diameter of SPLP was measured by quasi-elastic light scattering (QELS) using a Nicomp Model 370 Sub-Micron particle sizer (Santa Barbara, CA, USA) operated in the particle mode.

Clearance, biodistribution and tumor accumulation of SPLP

Lewis lung carcinoma cells (300 000; ATCC CRL-1642) were implanted subcutaneously in the hind flank of 6-week-old female C57BL/6 mice (Harlan, Indianapolis, IN, USA) and the tumor allowed to grow to approximately 200 mg (12–14 days). Injected materials were then administered intravenously (lateral tail vein injection). All injected doses are reported in micrograms of plasmid DNA per mouse. Blood from animals was collected at the appropriate time-points into blood collection tubes by cardiac puncture. Tumors and organs were quickly removed and frozen at –70°C. Aliquots of plasma separated from blood were analyzed for ^{3}H-CHE by liquid scintillation counting. Plasmid DNA was purified by treating 50 μl plasma with 1× proteinase K buffer (1.0 mg/ml proteinase K, 100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5% SDS, pH 8.0). After incubation at 37°C for 3 h, the samples were purified by phenol/chloroform extraction followed by ethanol precipitation. Tumors were homogenized in PBS containing 100 mM EDTA pH 8.0 by using the Fast-Prep 120 homogenizer system (Bio 101, Vista, CA, USA). DNA was purified from an aliquot of the tumor homogenates using the DNAzol reagent according to the manufacturer's guidelines (Life Technologies, Bethesda, MD, USA). The DNA preparations from tumor homogenates were digested with *Eco*RI. DNA samples were subject to electrophoresis through 1.0% agarose gels, transferred to nylon membranes and subjected to Southern blot hybridization using a random primed ^{32}P-labeled restriction fragment from the luciferase gene. Hybridization intensity was quantified using a STORM840 phosphor-imager (Molecular Dynamics, Sunnyvale, CA, USA) and converted to mass of DNA using a standard curve constructed with known amounts of plasmid DNA.

Luciferase assays

Tumor tissue was homogenized in 1× Cell Culture Lysis Reagent (CCLR) (Promega, Madison, WI, USA) using the Fast-Prep 120 homogenizer system (Bio 101). The homogenates were centrifuged at 10 000 *g* for 2 min before 20 μl of the supernatant was assayed for luciferase activity using the Luciferase Assay System Kit (Promega) on an ML3000 microtiter plate luminometer (Dynex Technologies, Chantilly, VA, USA). Luciferase activities were converted to mass quantities of purified luciferase by comparison with a standard curve generated by assaying known amounts of purified *Photinus pyralis* luciferase enzyme (Boehringer-Mannheim, Laval, PQ, Canada) diluted into untreated tumor extract.

Hepatic release enzyme assays

Plasma from normal C57BL/6 mice injected with SPLP, plasmid DNA–cationic lipid complexes or HBS was recovered 24 h after injection by centrifugation and assayed immediately for ALT or ASP using commercially available kits (Sigma, St Louis, MO, USA).

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JOINT APPENDIX 43

GENE DELIVERY

Synthesis and characterization of novel poly(ethylene glycol)-lipid conjugates suitable for use in drug delivery

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Abstract

Liposomal formulations have been used to encapsulate and deliver a wide variety of therapeutic and diagnostic agents. Their circulation can be prolonged by the addition of neutral, hydrophilic polymers such as poly(ethylene glycol) (PEG) to the outer surface. An extended circulation lifetime allows them to take advantage of the enhanced permeability and retention effect (EPR), resulting in increased delivery to target sites. Incorporation of PEG also prevents aggregation and aids in the formation of uniform, small mono-disperse particles. This is often accomplished with the use of PEG-lipid conjugates, PEG molecules with a hydrophobic domain to anchor them into the liposomal bilayer upon formulation.

Here we present data showing that some commonly used PEG-lipids are chemically unstable due to the presence of carboxylic ester bonds. This instability limits their utility in aqueous environments common to many liposomal preparations. To address this problem, we designed and synthesized three alternative PEG-lipids. Using SPLP (PEG-stabilized liposomal vesicles encapsulating plasmid DNA) as a model system, we investigated the properties of the novel PEG-lipids. An accelerated stability study was conducted at 37 °C for 42 days to confirm chemical stability and an in vivo model was used to assess the pharmacokinetics, toxicity and activity of the SPLP. We show that the novel PEG-lipids are more stable in liposomal formulation, less toxic upon systemic administration, and accordingly, are suitable replacements for the PEG-lipids described previously.

1. Introduction

Through liposomal encapsulation, it is possible to obtain particles with the small size, low surface charge and increased serum stability necessary to increase circulation lifetime and take advantage of the "enhanced permeation and retention" (EPR) effect [1–3]. This effect occurs where fenestrated vasculature permits the extravasation and increased accumulation of encapsulated material at target sites such as tumors, sites of infection or inflammation [4–6].

Nucleic acid based drugs acquire other benefits from liposomal encapsulation [7–11]. When administered systemically in unprotected form, nucleic acids suffer from poor pharmacokinetics due to rapid degradation by intravascular nucleases. Early liposomal strategies utilized lipoplex, electrostatic complexes formed between cationic lipids and negatively charged nucleic acids [12]. However, unshielded cationic lipoplexes themselves possess poor physico-chemical characteristics for systemic delivery. Their positive surface charge leads to non-specific interaction with anionic species in the blood, resulting in rapid clearance by the reticulo-endothelial system (RES) [13–15].

This problem can be overcome through the use of hydrophilic polymers attached to the particle's surface. Most commonly PEG has been used [16,17], although other polymers have been described [18,19]. However, it has been shown that the presence of PEG can affect the intracellular delivery and trafficking of non-viral vectors, resulting in lower gene expression [20]. To this end, many groups have devised strategies to ensure that the presence of PEG is transient. One strategy involves the use of exchangeable PEG-lipids that rely on slow diffusion from the particle surface at a rate determined by the size of their lipid anchors [9,21]. Examples include PEG-

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phosphatidyl ethanolamines (PEG-PE) [22], PEG-Ceramides (PER-Cer) [21], SAINT-PEGs [23] and PEG-succinoyl diacylglycerols (PEG-S-DAGs) [9]. Another strategy uses PEG-disulphide lipids that possess a disulphide bond between the polymer and the lipid anchor that is cleaved by thiolytic agents in the reductive environment of the endosome [24,25]. Similarly, vinyl ether PEG-lipids [26] and orthoester PEG-lipids [27–29] have been developed using chemical linkages that are sensitive to the reduced pH of the endosomal compartment.

Recently published data shows that when administering repeated doses of liposomally encapsulated, immunostimulatory nucleic acids, a strong, long-lived antibody response can be generated against PEG, a result of the powerful adjuvant effect of the nucleic acid payload [30,31]. While this effect has in the past been attributed to the immunostimulatory CpG motifs of bacterial pDNA [32], CpG free phosphorothioate ODN [33] and siRNA [34] have also been shown to be immunostimulatory. This impacts the potential for repeat administration of PEGylated nucleic acid delivery systems, causing a loss of disease site targeting, accelerated blood clearance and acute hypersensitivity upon subsequent administration. It has been shown that the use of PEG-lipids with a smaller C_{14} lipid anchor, thereby increasing the rate of dissociation of the PEG from the particle, abrogates this deleterious effect [30,31]. In this regard, there may be advantages to a strategy using exchangeable PEG layers, rather than those that are permanently bound or not cleaved until entry to the endosome.

Our characterization of the immune response to PEG-lipids [31] coincided with our observation that PEG-succinoyl distearylglycerol (PEG-S-DSG)-containing SPLP in a controlled, long-term stability study exhibited a steadily decreasing concentration of PEG-lipid over time (Fig. 1), ultimately leading to particle destabilization and aggregation. This led us to believe that PEG-S-DSG was chemically unstable, an undesirable attribute for a component of a potential pharmaceutical product. An examination of commercially available alternatives and the literature yielded no acceptable substitutes for PEG-S-DSG. Therefore, we set out to design, synthesize and characterize novel replacements with the aim of preparing SPLP that are at least as efficacious as those containing the original PEG-S-DSG.

2. Materials and methods

2.1. Materials and analyses

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). 1,2-dioleyloxy-*N,N*-dimethyl-3-aminopropane (DODMA) [35], PEG-S-DAGs [9] and PEG-CerC$_{20}$ [21] were prepared as previously described. ^{3}H-labelled CHE was obtained from Perkin-Elmer (Boston, MA, USA). The Picogreen Quantitation Assay and Kit was obtained from Molecular Probes (Eugene, OR, USA). GPR grade solvents were purchased from VWR Scientific (Edmonton, AB, Canada). All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada). The pCMVluc plasmid, encoding the luciferase reporter gene under

the control of the cytomegalovirus promoter, was manufactured as described previously [36]. ^{1}H nuclear magnetic resonance (NMR) spectrometry was performed by Spectral Data Services, Inc. (IL, USA). Elemental analysis (CHN) was performed by Canadian Microanalytical Service Ltd. (BC, Canada).

2.2. SPLP preparation

SPLP at a total lipid concentration of approximately 10 mg/mL were prepared using the method of spontaneous vesicle formation by ethanol dilution, as described previously [37]. The lipid composition was DSPC : cholesterol : PEG-lipid : DODMA (20:55:10:15 molar ratio). Nucleic acid encapsulation was determined using a PicoGreen assay, and encapsulation efficiency calculated by comparing fluorescence in the presence and absence of Triton X-100 [37]. Picogreen fluorescence was measured using a Varian Eclipse Spectrofluorometer (Varian Inc., CA, USA). Particle size was determined using a Malvern Instruments Zetasizer 3000HSA (Malvern, UK). SPLP (40 μL) were diluted with 4 mL of phosphate buffered saline (PBS (150 mM NaCl, 10 mM Phosphate, pH 7.4 buffer)). Intensity-weighted, Gaussian distribution analysis was used to determine mean vesicle diameters and population standard deviations.
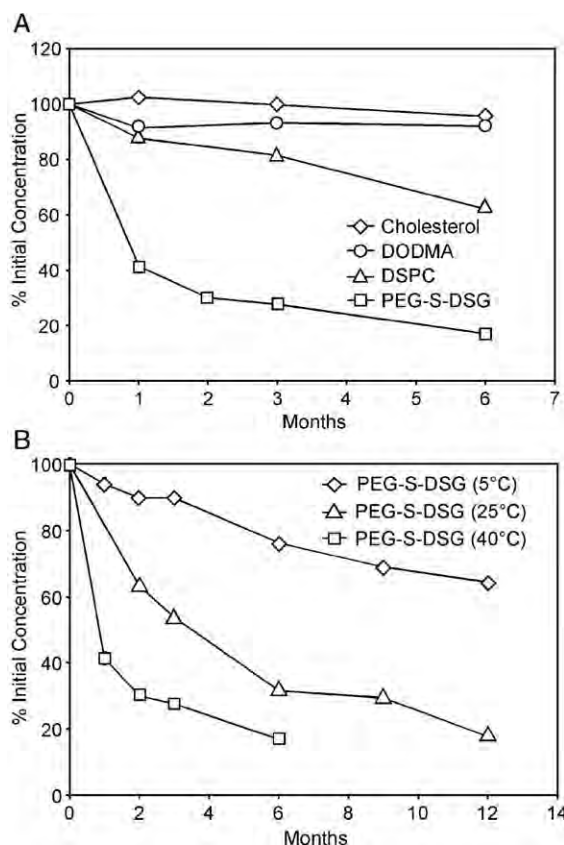

Fig. 1. (A) Accelerated stability study of SPLP lipids at 40 °C. Lipid concentrations were determined by HPLC analysis using an evaporative light scattering detector. PEG-S-DSG, possessing four carboxylic ester bonds, exhibited a pronounced tendency to degrade. DSPC was also unstable at 40 °C. (B) Stability study of PEG-S-DSG in SPLP at 40, 25 and 5 °C. Data-points are the mean of 2 analyses. Error shown is the percent relative standard deviation of six analyses of a standard containing all four lipids.

Polydispersity was reported using 'Contin' mode of the instruments PCS software.

2.3. SPLP stability studies and HPLC analysis

SPLP formulations were incubated at relevant temperatures (5, 25, 37 or 40 °C) for the duration of the study in question (up to 12 months for the long-term study, 42 days for the accelerated study). Samples (100 µL) were withdrawn using a Gilson pipette and prepared for HPLC analysis by diluting 1:19 in ethanol. This dissolved the SPLP lipid bilayer and resulted in precipitation of the nucleic acids and buffer salts. Precipitates were removed by filtering through a 13 mm Acrodisc 0.45 µm syringe filter (Pall Corp., Ann Arbor, MI). A Waters Alliance 2695 HPLC (Waters Ltd., ON, Canada) with an ACE C_8, 4.6×250 mm, 5 µm column (Canadian Life Science, ON, Canada), was used for analysis with sample injection volumes of 20 µL. Sample and column temperatures were 20 and 40 °C, respectively. The mobile phase gradient varied linearly from 80:20 A:B to 83:17 A:C (where A = methanol, B = 10 mM ammonium bicarbonate, pH 8.0, C = tetrahydrofuran (THF)) over a time period of 18 min. The mobile phase flow rate was 1.1 mL per min. A 6-min hold at the final conditions was followed by a 21-min re-equilibration period for the column (equating to a total run time of 45 min). An Alltech Evaporative Light Scattering Detector 2000 (Alltech Associates Inc., IL, USA) was used for detection, with the drift tube temperature set at 70 °C and gas flow rate set at 1.8 L per min. A standard curve was generated for each lipid. System suitability was determined prior to each run by analysis of a standard sample containing all four lipids. When the relative standard deviation (RSD) of six repeat analyses was less than 2.0% for each lipid, the run was considered acceptable. For the long-term stability study, $n=2$. For the accelerated study, $n=3$.

2.4. In vivo transfection studies

Neuro-2a neuroblastoma cells were cultured in Minimum Essential Medium (MEM; Invitrogen, ON, Canada), supplemented with 10% fetal bovine serum (FBS; Invitrogen, ON, Canada) at 37 °C with 5% CO_2. 5-week-old male A/J mice (Harlan, IN, USA) were inoculated subcutaneously in the hind flank with 1.5×10^6 Neuro-2a cells 13 days prior to SPLP treatment. SPLP containing 50 µg DNA in a 100 µL injection volume or 100 µL PBS were administered by lateral tail vein injection. Mice were sacrificed 48 h after treatment. Tumor, liver, lungs, spleen and heart tissues were subject to analysis for luciferase gene expression as described previously [9]. For the experiment in Fig. 5, $n=6$. For the experiment in Fig. 7B, $n=4$.

2.5. Toxicity studies

5-week-old male A/J mice (Harlan, IN, USA) were treated with SPLP containing 50 µg DNA in a 100 µL injection volume or 100 µL PBS, administered by lateral tail vein injection. Animals were sacrificed 48 h after treatment. Serum was assayed for alanine aminotransferase (ALT) and aspartate amiotransferase

(AST) by the Central Laboratory for Veterinarians (Langley, BC, Canada). For this experiment, $n=3$.

2.6. Pharmacokinetic studies

SPLP containing tritiated cholesteryl hexadecyl ether (^{3}H-CHE) (1.0 µCi/mg of total lipid) in the lipid solution were prepared as in Section 2.2. Injections containing 50 µg DNA in a 100 µL volume were administered to 5-week-old male A/J mice (Harlan, IN, USA) by lateral tail vein injection. At appropriate time points, ^{3}H-CHE in whole blood was determined by liquid scintillation counting, using Picofluor 15 and a Beckman LS6500 (Beckman Instruments, CA, USA). For this experiment, $n=4$.

2.7. Synthesis of lipid anchors

The synthesis of the C_{18} lipid anchor is described below. The C_{14} anchor was made in an analogous fashion, substituting an equimolar amount of 1-bromotetradecane for the 1-bromooctadecane in the reaction to make the analogous compound **1**.

2.7.1. Preparation of 1,2-distearyloxy-3-allyloxypropane (1)

Benzene (250 mL) was added to 95% sodium hydride (11.4 g, 450.0 mmol), and the flask was sealed and flushed with nitrogen. A solution of 3-allyloxy-1,2-propanediol (6.6 g, 50.0 mmol) in benzene (75 mL) was added to the flask. 96% 1-bromooctadecane (41.7 g, 120.0 mmol) was added and the reaction left to reflux overnight under nitrogen. The mixture was cooled to room temperature and excess sodium hydride slowly quenched with ethanol. The solution was transferred to a separatory funnel with benzene (250 mL) and washed with distilled water (2×200 mL) and brine (1×200 mL). The organic fractions were combined, dried over magnesium sulfate and concentrated. The crude product was purified by flash column chromatography (1–5% ether in hexane) to yield compound **1** as a colourless wax (21.0 g, 66%, $R_f=0.35$ (5% ether in hexane)). ^{1}H NMR (400 MHz), δ_H: 5.90 (m, 1H, $CH_2=CH$), 5.21 (m, 2H, $CH_2=CH$), 4.01 (m, 2H, $CH_2=CHCH_2$), 3.61–3.40 (m, 9H, $CH_2CH(OCH_2)CH_2OCH_2$), 1.61–1.51 (m, 4H, OCH_2CH_2), 1.38–1.20 (m, 60H, $CH_{2(stearyl)}$), 0.88 (t, 6H, CH_3, $J=6.8$ Hz).CHN found; C 79.06, H 13.15 ($C_{42}H_{84}O_3=C$ 79.18; H 13.29; O 7.53).

2.7.2. Preparation of 1,2-distearyloxypropan-3-ol (2)

Compound **1** (21.0 g, 33 mmol) was dissolved in ethanol (250 mL) and trifluoroacetic acid (20 mL) and tetrakis (triphenylphosphine) palladium(0) (5.0 g, 4.3 mmol) added. The reaction mixture was refluxed under nitrogen overnight. The solvent was removed by rotary evaporator and the crude product purified by flash column chromatography (100% dichloromethane (DCM)), to yield compound **2** as a colourless wax (18.7 g, 95%, $R_f=0.4$ (chloroform)). ^{1}H NMR (400 MHz), δ_H: 3.75–3.67 (m, 1H, OCH), 3.67–3.47 (m, 8H, OCH_2), 2.26 (s, 1H, OH), 1.62–1.50 (m, 4H, OCH_2CH_2), 1.38–1.20 (m, 60H, $CH_{2(stearyl)}$), 0.88 (t, 6H, CH_3, $J=6.8$ Hz). CHN found; C 79.06, H 13.25 ($C_{39}H_{80}O_3=C$, 78.46; H, 13.51; O, 8.04).

2.7.3. Preparation of N-(2,3-distearyloxypropyl)phthalimide (3)

97% methanesulphonic anhydride (11.3 g, 62.8 mmol) was dissolved in DCM (anhydrous, 100 mL) and pyridine (anhydrous, 5.0 g, 62.8 mmol) slowly added. A solution of compound **2** (18.7 g, 31.4 mmol) in DCM (anhydrous, 100 mL) was added and the reaction stirred overnight at room temperature. The reaction mixture was washed with distilled water (2×200 mL) and brine (1×200 mL), dried over magnesium sulphate, and concentrated to yield the mesylate as a colourless wax ($R_f = 0.8$ (chloroform)). The mesylate and potassium phthalimide (15.7 g, 85.0 mmol) were added to N,N-dimethylformamide (DMF) (anhydrous, 500 mL) and the mixture stirred at 70 °C overnight under nitrogen. DMF was removed by rotary evaporator attached to a high vacuum pump and the residue triturated in chloroform (300 mL). The suspension was filtered and the filtrate washed (distilled water (2×200 mL) and brine (1×200 mL), dried (magnesium sulphate) and concentrated. The crude product was purified by flash column chromatography (100% DCM) to yield compound **3** as a colourless wax (19.5 g, 86%, $R_f = 0.65$ (DCM)). ^{1}H NMR (400 MHz), δ_H: 7.85 (dd, 2H, C$H_{(arom\ 3,\ 6)}$, $J_{ortho} = 5.5$ Hz, $J_{meta} = 3.0$ Hz), 7.71 (dd, 2H, C$H_{(arom\ 4,\ 5)}$), 3.90–3.70 (m, ^{3}H, NCH_2CH), 3.63–3.38 (m, 6H, OCH_2), 1.54–1.37 (m, 4H, OCH$_2$CH_2), 1.38–1.06 (m, 60H, C$H_{2(stearyl)}$), 0.88 (t, 6H, CH_3, $J = 6.9$ Hz). CHN found; C 77.76, H 11.69, N 1.86 ($C_{47}H_{83}NO_4 = $C 77.74, H 11.52, N 1.93, O 8.81).

2.7.4. Preparation of 1,2-distearyloxypropyl-3-amine (4)

Phthalimide deprotection was performed as described previously [38]. Briefly, compound **3** (19.5 g, 26.9 mmol) was dissolved in ethanol (500 mL), hydrazine monohydrate (40 mL, 825 mmol) added and the reaction refluxed overnight. A pale yellow precipitate developed so the suspension was filtered and the filtrate evaporated under reduced pressure. The crude product was purified by flash column chromatography (0–5% MeOH–CHCl$_3$) to give compound **4** as a pale yellow wax (13.1 g, 82%, $R_f = 0.45$ (8% methanol (MeOH) in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 3.65–3.58 (m, 1H, NCH$_2$CH), 3.52–3.32 (m, 6H, OCH_2), 2.79 (ABX, 2H, NCH_2 $_{(A\ and\ B)}$, $J_{AB} = 13.2$ Hz, $J_{AX} = 3.7$ Hz, $J_{BX} = 6.4$ Hz), 1.62–1.51 (m, 4H, OCH$_2$CH_2), 1.40–1.20 (m, 62H, NH_2, C$H_{2(stearyl)}$), 0.88 (t, 6H, CH_3, $J = 6.8$ Hz). CHN found; C 78.69, H 13.42, N 2.66 ($C_{39}H_{81}NO_2 = $C, 78.58; H, 13.70; N, 2.35; O, 5.37).

2.8. Synthesis of PEG-lipids

2.8.1. Preparation of methoxy PEG$_{2000}$ acetic acid (5)

Methoxy poly(ethylene glycol)$_{2000}$ (Me-PEG$_{2000}$-OH) (20.0 g, 10 mmol) was added to a solution of sodium dichromate (3.0 g, 10 mmol) in 10% sulfuric acid (200 mL) and stirred at room temperature overnight. The product was extracted with chloroform (3×250 mL) and the organic fraction combined, washed with 1 M sodium hydroxide (250 mL) and evaporated to yield a pale blue wax. This crude material was purified by flash column chromatography (0–15% MeOH–CHCl$_3$) to give compound **5** as a colourless solid (8.0 g, 38.7%, $R_f = 0.3$ (13% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 4.0–3.9 (s, 2H, CH_2CO$_2$H), 3.7–3.6 (m, ~180H, OC$H_{2(PEG)}$), 3.38 (s, ^{3}H, CH$_3$O).

2.8.2. Preparation of N-(1,2-distearyloxypropyl) methoxy poly (ethylene glycol)$_{2000}$ acetamide (PEG-A-DSA) (6)

Compound **5** (6.8 g, 3.4 mmol) was dissolved in benzene (80 mL) and oxalyl chloride (3.4 mL, 20 mmol) slowly added. The solution was stirred for 2 h prior to solvent removal by rotary evaporator. Compound **4** (2.15 g, 3.6 mmol), DCM (anhydrous, 80 mL) and triethylamine (TEA) (3 mL, 20 mmol) were added and the reaction was stirred for 48 h. The solution was acidified via the addition of a 1% solution of hydrochloric acid (HCl) (250 mL) with agitation. The organic layer was collected, dried over magnesium sulphate and concentrated to yield a pale yellow solid. The crude product was purified by flash column chromatography (0–7% MeOH in CHCl$_3$), then lyophilized to yield PEG-A-DSA as a colourless solid (3.4 g, 38%, $R_f = 0.5$ (10% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 7.10–7.04 (m, 1H, NH), 4.00 (s, 2H, CH_2C(O)NH), 3.7–3.6 (m, ~180H, OC$H_{2(PEG)}$), 3.60–3.39 (m, 9H, NCH_2CH(OCH_2)CH_2OCH_2), 3.38 (s,^{3}H, CH$_3$O), 1.60–1.50 (m, 4H, OCH$_2$C$H_{2(stearyl)}$), 1.38–1.20 (m, 60H, C$H_{2(stearyl)}$), 0.88 (t, 6H, CH$_2$CH_3, $J = 6.8$ Hz).

2.8.3. Preparation of methoxy poly(ethylene glycol)$_{2000}$ mesylate (ME-PEG$_{2000}$-OMs) (7)

Pyridine (3.8 mL, 47.0 mmol) was added slowly to a solution of 97% methanesulphonic anhydride (8.2 g, 47.1 mmol) in DCM (anhydrous, 80 mL). A solution of Me-PEG$_{2000}$-OH (31.5 g, 15.5 mmol) in DCM (anhydrous, 120 mL) was added and the reaction stirred overnight. The reaction mixture was washed with distilled water (2×200 mL) and brine (1×200 mL), dried over magnesium sulphate, and concentrated. Purification by flash column chromatography (0–10% MeOH–CHCl$_3$) yielded compound **7** as a colourless solid (30.1 g, 92.8%, $R_f = 0.4$ (5% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 4.38 (t, ^{3}H, CH_2OS, $J = 4.5$ Hz), 3.79–3.74 (m, 2H, CH_2CH$_2$OS), 3.7–3.6 (m, ~180H, OC$H_{2(PEG)}$), 3.38 (s, ^{3}H, CH$_3$O), 3.08 (s, ^{3}H, OS(O)$_2$CH_3).

2.8.4. Preparation of 1-amino methoxy poly(ethylene glycol) (Me-PEG$_{2000}$-NH$_2$) (8)

Me-PEG$_{2000}$-NH$_2$ was prepared as previously described [39]. Briefly, compound **7** (10 g, 5 mmol) was dissolved in a concentrated solution of aqueous ammonia (400 mL), sealed and left to stir for 72 h. The product was extracted with DCM (3×300 mL) and the combined organic fractions dried over MgSO$_4$ and concentrated. The product was crystallized from diethyl ether to yield compound **9** as a colourless solid (9.3 g, 93%, $R_f = 0.15$ (10% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 3.7–3.6 (m, ~180H, OC$H_{2(PEG)}$), 3.58–3.53 (m, 2H, CH_2CH$_2$NH$_2$), 3.38 (s, ^{3}H, CH$_3$O), 2.90 (t, 2H, CH_2NH$_2$, $J = 5.2$ Hz), 2.2–1.9 (bs, 2H, NH$_2$).

2.8.5. Preparation of methoxy poly(ethylene glycol) succinimide (Me-PEG$_{2000}$-Sn) (9)

Succinic anhydride (3.8 g, 38.1 mmol) was added to a solution of compound **8** (9.0 g, 4.4 mmol) in pyridine (anhydrous, 100 mL)

and the reaction stirred overnight. The pyridine solvent was removed under reduced pressure, and the residue dissolved in distilled water (100 mL) and acidified with HCl. Crude product was extracted with DCM (3×100 mL), dried over magnesium sulphate and concentrated. Purification by flash column chromatography (0–10% MeOH–CHCl$_3$) yielded compound **9** as a colourless solid (5.7 g, 61%, R_f=0.3 (10% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 6.69 (m, 1H, N*H*), 3.7–3.6 (m, ~180H, OC*H*$_{2(PEG)}$), 3.58–3.53 (m, 2H, C*H*$_2$CH$_2$NH), 3.45 (quint, 2H, CH$_2$C*H*$_2$NH, J=5.0 Hz), 3.38 (s, ^{3}H, CH$_3$O), 2.70–2.61 (m, 2H, C*H*$_2$CO$_2$H), 2.57–2.50 (m, 2H, C*H*$_2$CH$_2$CO$_2$H).

2.8.6. Preparation of N-[(methoxy poly(ethylene glycol)$_{2000}$) succinimidyl]-1,2-distearyloxlpropyl-3-amine (PEG-S-DSA) (10)

N-hydroxysuccinamide (360 mg, 3 mmol) and compound **9** (3.1 g, 1.5 mmol) were dissolved in chloroform (anhydrous, 30 mL). A solution of 1,3-dicyclohexyl-carbodiimide (DCC) (490 mg, 2.25 mmol) in chloroform (anhydrous, 10 mL) was added, and the reaction stirred for 1 h. A separate solution of compound **4** (900 mg, 1.5 mmol) and TEA (0.9 mL, 6 mmol) in chloroform (anhydrous, 10 mL) was added and the reaction for a further hour. The solution was filtered through a bed of Celite and distilled water added (50 mL). The mixture was acidified with concentrated HCl, then washed again with distilled water (2×50 mL) and brine (50 mL). Organic fractions were combined, dried over magnesium sulphate and concentrated. The product was purified by flash column chromatography (0–7% MeOH–CHCl$_3$) to yield PEG-S-DSA, compound **10**, as a colourless solid (3.5 g, 88%, R_f=0.65 (12% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 6.84–6.79 (m, 1H, N*H*CH$_2$CH), 6.40–6.34 (m, 1H, CH$_2$CH$_2$N*H*), 3.7–3.6 (m, ~180H, OC*H*$_{2(PEG)}$), 3.60–3.39 (m, 13H, OC*H*$_2$CH$_2$CN, NC*H*$_2$C*H*(OC*H*$_2$)C*H*$_2$OC*H*$_2$), 3.38 (s, 3H, CH$_3$O), 2.54–2.49 (m, 2H, C*H*$_2$CH$_2$CO$_2$H), 1.60–1.51 (m, 4H, OCH$_2$C*H*$_{2(stearyl)}$), 1.40–1.20 (m, 60H, C*H*$_{2(stearyl)}$), 0.88 (t, 6H, C*H*$_3$, J=6.9 Hz).

2.8.7. N-[(methoxy poly(ethylene glycol)$_{2000}$)carbamyl]-1,2-distearyloxlpropyl-3-amine (PEG-C-DSA) (11)

Diphosgene (2.0 mL, 16.7 mmol) was added to a solution of PEG$_{2000}$ methyl ether (10.0 g, 5 mmol) in DCM (anhydrous, 100 mL) and stirred under nitrogen at room temperature for 3 h. DCM and excess diphosgene were then removed under reduced pressure and compound **4** (4.2 g, 7 mmol) added. The flask was flushed with nitrogen, then DCM (anhydrous, 150 mL) and TEA (1.4 mL) added prior to stirring overnight. The solution was diluted with DCM (100 mL), washed (1% HCl (1×200 mL), water (1×200 mL) and brine (1×200 mL)), dried over magnesium sulphate and concentrated. Purification by flash column chromatography (1.5–6.0% MeOH in CHCl$_3$) followed by lyophilization afforded PEG-C-DSA, compound **11**, as a colourless solid (11.9 g, 90%, R_f=0.35 (8% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 5.16–5.08 (m, 1H, N*H*), 4.25–4.19 (m, 2H, C*H*$_2$OC(O)), 3.7–3.6 (m, ~180H, OC*H*$_{2(PEG)}$), 3.60–3.39 (m, 9H, NC*H*$_2$C*H*(OC*H*$_2$)C*H*$_2$OC*H*$_2$), 3.38 (s, 3H, CH$_3$O), 1.60–1.50 (m, 4H, OCH$_2$C*H*$_{2(stearyl)}$), 1.38–1.20 (m, 60H, C*H*$_{2(stearyl)}$), 0.88 (t, 6H, CH$_2$C*H*$_3$, J=6.8 Hz).

2.8.8. N-[(methoxy poly(ethylene glycol)$_{2000}$)carbamyl]-1,2-dimyristyloxlpropyl-3-amine (PEG-C-DMA) (12)

PEG-C-DMA, compound **12**, was synthesized in a manner similar to PEG-C-DSA, compound **11**, on a 5 mmol scale to yield, after chromatography, compound **12** as a colourless solid (11.1 g, 88%, R_f=0.35 (8% MeOH in CHCl$_3$)). ^{1}H NMR (400 MHz), δ_H: 5.21–5.15 (m, 1H, N*H*), 4.26–4.19 (m, 2H, C*H*$_2$OC(O)), 3.7–3.6 (m, ~180H, OC*H*$_{2(PEG)}$), 3.60–3.39 (m, 9H, NC*H*$_2$C*H*(OC*H*$_2$)C*H*$_2$OC*H*$_2$), 3.38 (s, ^{3}H, CH$_3$O), 1.60–1.50 (m, 4H, OCH$_2$C*H*$_{2(myristyl)}$), 1.38–1.20 (m, 44H, C*H*$_{2(myristyl)}$), 0.88 (t, 6H, CH$_2$C*H*$_3$, J=6.9 Hz).

3. Results

3.1. PEG-S-DSG is chemically unstable in liposomal formulations

To ascertain lipid stability, we studied lipid concentrations over time in SPLP samples stored at different temperatures. Formulations containing the lipids DSPC : cholesterol : PEG-S-DSG : DODMA (20:55:10:15 molar ratio) were examined by HPLC for degradation at 5, 25 and 40 °C. DODMA and cholesterol exhibited negligible structural instability, even under accelerated conditions (40 °C, Fig. 1A), while DSPC degraded to a certain extent. However, PEG-S-DSG degraded rapidly, with more than 10% of the lipid degrading in the first 2 months, even at 5 °C (Fig. 1B). As expected the instability of PEG-S-DSG was exacerbated at higher temperatures. The PEG-S-DSG half-life in aqueous liposomal formulations was 18.3, 5.2 and 2.7 months at 5, 25 and 40 °C, respectively. Half-life values were calculated using a first order, non-compartmental model. Log values of the concentrations were plotted against time to give straight lines, the slopes of which were used to calculate half-life.

3.2. Synthesis of novel PEG-lipids

Three novel PEG-lipids were synthesized for consideration as potential replacements for PEG-S-DSG; PEG-S-DSA, PEG-A-DSA and PEG-C-DSA. Each used a different linker to join the PEG to the lipid anchor (the previously described 1,2-distearyloxypropyl-3-amine (DSA) [38]); PEG-S-DSA, PEG-A-DSA and PEG-C-DSA utilized succinimide, amide and carbamate linkers, respectively (Fig. 2). The structure of DSA allows the possibility of synthesizing analogues with shorter alkyl chains, necessary for giving the PEG-lipids the desired programmable, exchangeable properties. The synthetic route to the lipids was comprehensively revised to allow more efficient manufacture, such that the overall yield was improved by approximately 50% while the number of synthetic steps was decreased from six to four. The synthesis and PEG-coupling processes (Fig. 3) displayed varying efficiency. Although the 2-step strategy used to obtain PEG-A-DSA had a low overall yield of 14%, this did not present a problem as the reactions are easily performed on large scale. PEG-S-DSA predictably had a low yield also (46%), having the longest synthetic route with 4 discrete steps. The PEG-C-DSA single-step synthesis was least complicated, and, unsurprisingly, had the highest yield of 90%. All synthetic products were
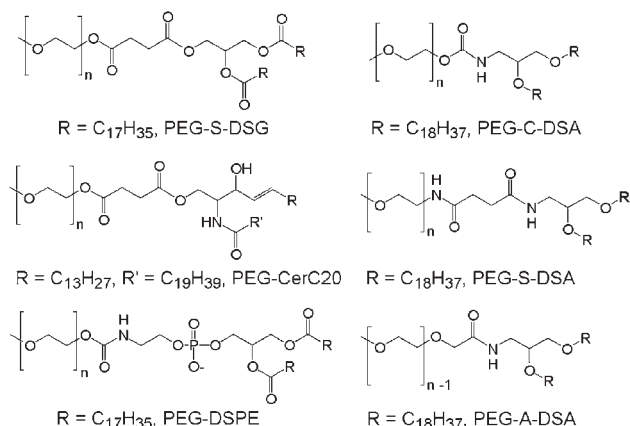
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Fig. 2. Chemical structures of the 2000 molecular weight ($n=45$) PEG-lipids assessed as replacements for PEG-S-DSG (top left). The 3 novel lipids (right hand column) were designed without carboxylic ester bonds present in the readily available alternatives (left column).

analyzed by TLC and gave single spots with expected R_f values. They were further characterized by ^{1}H NMR and, in the case of non-polymeric compounds, elemental analysis.

3.3. Physical characterization of formulations

The formulation characteristics of the six PEG-lipids were evaluated with particular emphasis on particle size and encapsulation efficiency. The PEG-lipids were used to prepare SPLP by spontaneous vesicle formation [37]. The PEG-DSPE formulation had a mean particle size of 161 nm. All other formulations had the

characteristic small particle size of SPLP (approximately 120 nm diameter, Table 1). Encapsulation efficiency was also significantly less with the use of PEG-DSPE; 61% as compared to 72% to 80% for the other 5 formulations ($P<0.05$, t-test). Final DNA encapsulation values were all somewhat similar, as the SPLP were passed through Mustang Q cartridges to remove unencapsulated DNA at the end of the formulation process. Even so, PEG-DSPE resulted in a formulation with a significantly lower percentage of encapsulated nucleic acid in the final product, as compared to the other PEG-lipids ($P<0.05$, t-test).

3.4. The presence of a succinate linker promotes instability in PEG-lipids

Assessment of PEG-lipid stability within the vesicles was of interest. Therefore, formulations containing the six PEG-lipids were analyzed for lipid degradation in an accelerated stability study, carried out over a six-week period at 37 °C. As expected from the earlier results, PEG-S-DSG was shown to degrade steadily over the course of the experiment ($t_{1/2}=58$ days) and PEG-CerC$_{20}$ degraded at a very similar rate ($t_{1/2}=47$ days) (Fig. 4). The breakdown of both lipids was shown to be significant, even after 7 days ($P<0.05$, t-test). The remaining PEG-lipids exhibited no obvious signs of instability during the six-week time-course, with concentrations after 42 days not significantly different from initial values ($P>0.1$ in all cases, t-test). The other lipids, cholesterol, DSPC and DODMA, were also stable during this time period. It is interesting to note that PEG-DSPE, despite containing two carboxylic ester bonds in its structure, appears to be stable within the context of this particular experiment.

3.5. In vivo gene expression from intravenous administration of SPLP

It has previously been shown that use of PEG-S-DSG or PEG-CerC$_{20}$ allows for the formation of long circulating particles that yield preferential gene expression in tumor tissue [8,9]. Ensuring a similar performance with the new PEG-lipids was of obvious importance. Therefore, gene expression was evaluated in a mouse tumor model following intravenous (i.v.) administration of SPLP containing the pCMVluc plasmid. 48 h
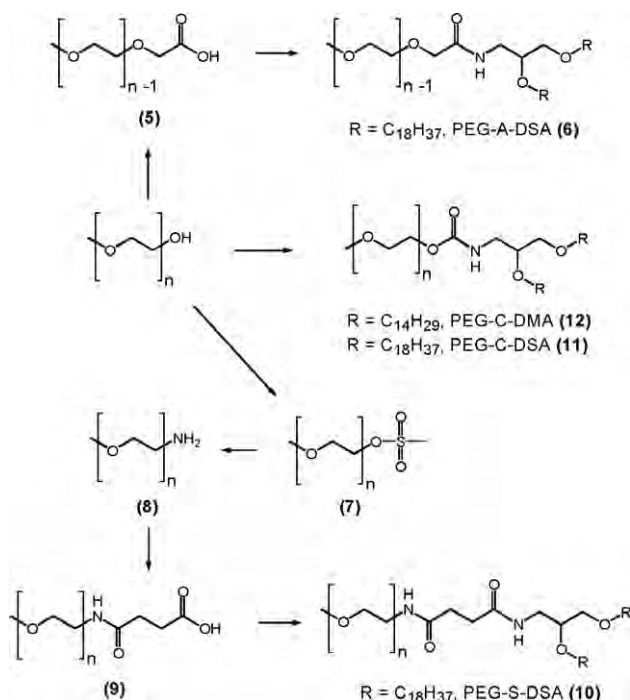

Fig. 3. Synthesis of three novel PEG-lipids. The PEG-lipids possessed carbamate, amide and succinimide linkers, rather than the succinate linker used in PEG-S-DSG and PEG-CerC$_{20}$.

Table 1
Physical properties of the SPLP formulations

PEG-lipid	Mean particle diameter (nm±S.D.)	Initial encapsulated DNA (%)	Final encapsulated DNA (%)	Charge of PEG-lipid at formulation pH (5.0)
PEG-S-DSG	118±4	80±1	94±1	Neutral
PEG-DSPE	161±5	61±3	84±2	−1
PEG-CerC$_{20}$	109±6	74±2	94±2	Neutral
PEG-A-DSA	122±5	72±2	89±2	Neutral
PEG-S-DSA	122±6	75±1	95±1	Neutral
PEG-C-DSA	118±5	72±2	89±1	Neutral
PEG-C-DMA	122±5	78±2	95±2	Neutral

Values are the mean of 3 separate experiments, the error stated is the standard deviation.
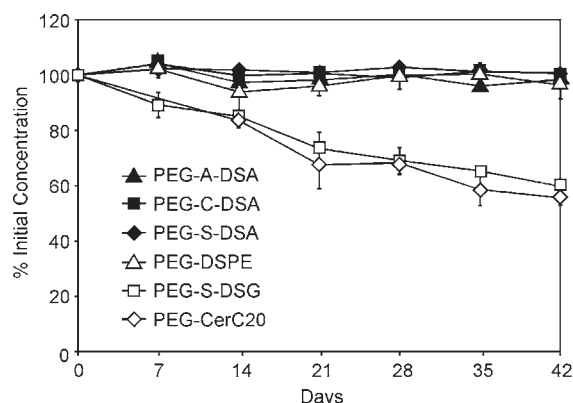
GENE DELIVERY

Fig. 4. Stability of PEG-lipids in SPLP at 40 °C. Lipid concentrations were determined by HPLC analysis. Error shown is standard deviation ($n=3$).

after SPLP administration, luciferase expression was evaluated in the liver, lung, spleen heart and tumor. As shown previously with PEG-S-DSG [9] or PEG-CerC$_{20}$ [40] containing SPLP, an excellent differential in luciferase protein levels was observed between the tumor and other tissues (Fig. 5). All six formulations were similar in this respect, typically resulting in tumor gene expression 2 orders of magnitude greater than that observed in other, non-target tissues.

3.6. Intravenous administration of SPLP is well tolerated

The relative toxicity of the new PEG-lipids was clearly of interest, as they are ultimately intended for in vivo applications. The toxicity following SPLP administration was examined by determining levels of the transaminases ALT and AST in mouse serum following SPLP treatment (Fig. 6). ALT is primarily regarded as a hepatocytes protein and increases in ALT are thought to indicate liver damage. AST is present in most tissues but particularly in cardiac muscle, skeletal muscle and the liver. Elevations in AST are regarded as a more general indication of systemic tissue damage. None of the formulations resulted in large increases in either enzyme, and ALT and AST levels actually remained within the normal limits for the PEG-A-DSA and PEG-

C-DSA SPLP. The remaining four formulations exhibited only marginally increased levels of either enzyme.

3.7. Circulation half-life of SPLP comprising PEG-lipids with shorter lipid anchors

Formulation, stability, transfection and toxicity data, combined with the ease of synthesis, lead to the selection of the carbamate linker chemistry for further characterization. The C$_{14}$ analogue (PEG-C-DMA) was synthesized to confirm that, similarly to PEG-S-DAGs and PEG-Ceramides, bilayer exchangeability (and thus pharmacology) could be modulated by varying the size of the lipid anchor. SPLP were prepared incorporating a ^{3}H-CHE-lipid marker. The percentage of injected dose remaining in circulation following a single injection of SPLP in the tail vein is displayed as a function of time (Fig. 7A). SPLP containing PEG-C-DSA exhibited a circulation half-life of 16 h, similar to that of the PEG-S-DSG containing formulation. SPLP containing PEG-C-DMA cleared more rapidly with a half-life of approximately 2 h. These results are very similar to those reported previously for SPLP containing the PEG-DAGs [9] and PEG-Ceramides [7]. The initial phase of the curve (from 0 to 8 h) was used to calculate half-life, using a first order, non-compartmental model. This initial phase was felt to be the most relevant part of the curve, as it indicates how quickly the majority of the SPLP dose is cleared. It also represents 4 out of the 5 data-points. Log values of the concentrations were plotted against time to give straight lines, the slopes of which were used to calculate half-life.

3.8. Gene expression patterns from formulations containing PEG-lipids with shorter lipid anchors

We have previously determined that the use of PEG-lipids with a shorter, C$_{14}$ lipid anchor is necessary to avoid the generation of an antibody response when delivering encapsulated nucleic acids [30,31]. The main drawback to this strategy is that the shorter lipid anchor leads to faster clearance, meaning less time for the
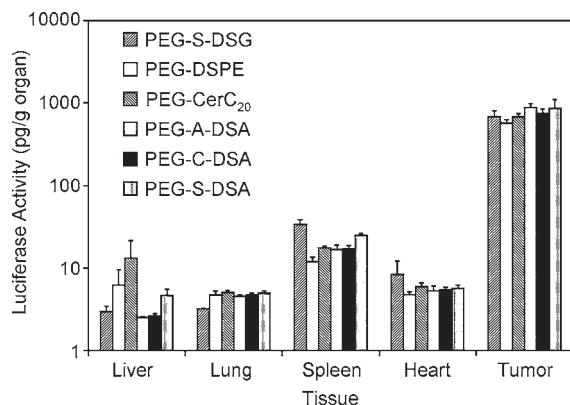

Fig. 5. Biodistribution of luciferase gene expression in Neuro-2a tumor-bearing male A/J mice. Gene expression was assessed 48 h after a single intravenous administration of SPLP. Error shown is standard deviation ($n=6$).
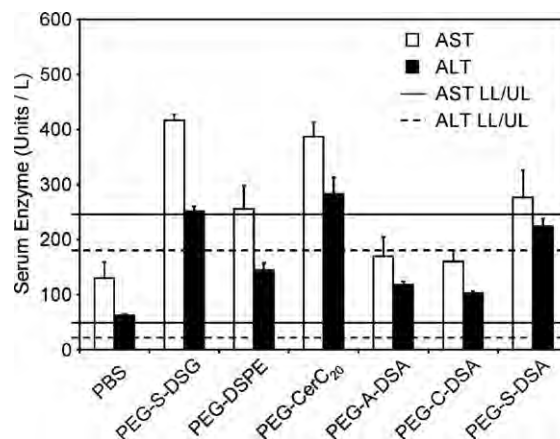

Fig. 6. Toxicity resulting from intravenous administration of SPLP. Serum was collected from mice and assayed for the enzymes alanine transferase (ALT) and aspartate transferase (AST). The upper and lower limits of historical normal values, as quoted by the Canadian Council on Animal Care, are indicated by dotted lines. Error bars represent standard deviation ($n=3$).
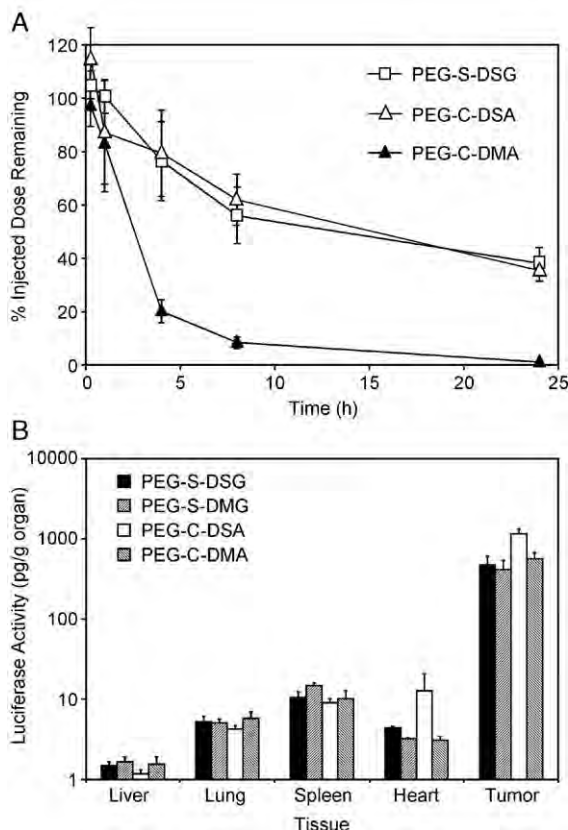
Fig. 7. (A) Plasma clearance of SPLP containing PEG-S-DSG, PEG-C-DSA or PEG-C-DMA. The percentage of injected dose remaining in plasma of mice following a single intravenous administration is displayed. SPLP were labelled with ^{3}H-cholesteryl hexadecyl ether (1 μCi per mg of lipid). Error bars represent standard deviation ($n=4$). (B) Biodistribution of gene expression resulting from administration of SPLP containing C_{14} or C_{18} PEG-lipid analogues. SPLP were administered by intravenous administration and gene expression measured 48 h later. Error shown is standard deviation ($n=4$).

particles to accumulate at the disease site and typically resulting in lower gene expression at distal tumor sites. It was of interest to evaluate to what degree this phenomenon affected gene expression using PEG-C-DMA SPLP. SPLP were prepared using the C_{14} (PEG-C-DMA and PEG-succinoyl dimyristylglycerol (PEG-S-DMG)) and C_{18} (PEG-C-DSA and PEG-S-DSG) analogues of PEG-C-DAAs and PEG-S-DAGs. Gene expression was evaluated in the liver, lung, spleen heart and tumor 48 h after a single intravenous administration of SPLP. Although tumor gene expression resulting from PEG-C-DMA SPLP treatment was significantly lower than that obtained with PEG-C-DSA ($P<0.05$), there was no significant difference between the level of tumor gene expression following treatment with PEG-S-DMG, PEG-S-DSG, or PEG-C-DMA SPLP (Fig. 7B). Consistent with previous results, a marked differential was seen between the gene expression in tumor tissue and the other tissues examined, typically of 2 orders of magnitude.

4. Discussion

While conducting long-term stability tests on SPLP, we discovered that the PEG-lipid, PEG-S-DSG, was unstable. Upon storage, even at 4 °C, more than 10% of the PEG-lipid was degraded within 2 months (Fig. 1). This effectively disqualifies this PEG-lipid from incorporation into any formulation to be considered for clinical development. We hypothesized that this instability was due to the presence of readily hydrolysable carboxylic ester bonds (of which there are 4 in the structure of PEG-S-DSG) [41,42]. Of the possible replacements, PEG-Ceramides [21] and PEG-DSPE possess ester bonds. PEG-DSPE also possesses an electrostatic charge, at both formulation and physiological pH, as do SAINT-PEGs [23]. This was also considered to be undesirable, for reasons of increased blood clearance [43,44], possible complement activation, and/or possible leakage of encapsulated material [21].

Another strategy for facilitating the intracellular delivery of PEG-lipid-containing particles is the use of PEG-lipids designed to cleave in the endosome. These include the disulphide-, vinyl ether- and diorthoester-linked PEG-lipids mentioned previously [24–29]. However, when considering the PEGylation strategy for nucleic acid containing particles, it is important to consider recent reports of an acquired immune response to C_{18} PEG-lipids with a long residence time on the particle [30,31]. The environmentally sensitive PEG-lipids described to date utilize C_{18} lipid anchors, and therefore may illicit similar immune responses when used to deliver immunostimulatory nucleic acids. The use of PEG-lipid linker chemistries that were designed to cleave in response to environmental triggers was rejected in favor of using exchangeable PEG-lipids.

We designed three novel PEG-lipids as potential replacements for PEG-S-DSG, each containing a different linker connecting the PEG and lipid domains. The first, PEG-S-DSA, utilized a succinimide linker, a direct (amide) analogue of the labile succinate linker in PEG-S-DSG. The yield for coupling of the PEG and lipid moieties was quite low, with an overall yield of 14%. The second and third PEG-lipids, PEG-A-DSA and PEG-C-DSA, possessed simple amide and carbamate linkers. These required fewer steps to synthesize and yields were much higher, 46% and 90%, respectively. All novel PEG-lipids utilized the same type of lipid anchor. Similarly to the linker, the carboxylic esters in the lipid anchor were replaced, in this case with ether bonds. The lipid possesses a primary amine head group to facilitate coupling to PEG. The dialkylglyceryl nature of the hydrophobic anchor was retained, to facilitate straightforward adjustment of the diffusible nature of the resulting PEG-lipid conjugate. When performing PEG-lipid coupling, an excess of lipid was used in relation to PEG. While this may seem counterintuitive (since the lipid anchor requires more synthetic steps to prepare than PEG), subsequent work up and purification of the final compounds was found to be easier with this approach. The C_{18} analogues of all PEG-lipids were synthesized initially to allow for direct comparison with PEG-S-DSG.

All of the PEG-lipids, with the exception of PEG-DSPE, were readily incorporated in SPLP and resulted in acceptable formulations. PEG-DSPE, being the only PEG-lipid to possess a negative charge, yielded larger particles (~160 nm) than the

GENE DELIVERY

five neutral compounds (~120 nm). The model for SPLP formation and nucleic acid encapsulation involves a charge interaction between nascent cationic lipid-containing bilayer fragments and the negatively charged nucleic acid. PEG-DSPE may act to partially neutralize the charge on the bilayer fragments or actively repel the DNA by charge repulsion, as encapsulation efficiency was also reduced when using PEG-DSPE.

Stability assessment of the individual PEG-lipids incorporated in SPLP yielded interesting results (Fig. 4). PEG-S-DSG was shown to degrade steadily in aqueous solution, as was the PEG-CerC$_{20}$. PEG-DSPE, however, is stable, despite possessing two carboxylic ester bonds similar to those thought to contribute to PEG-S-DSG instability. This may be explained by the location of the ester bonds. PEG-S-DSG and PEG-CerC$_{20}$ both contain ester bonds in the succinate linkers, between the PEG and hydrophobic domains of the molecule. These are expected to be located at the surface of the SPLP lipid bilayer, allowing ingress of water molecules necessary for the hydrolysis of this bond. Conversely, the ester bonds of PEG-DSPE are located in the hydrophobic domain of the molecule and would be expected to be sequestered deep within the lipid bilayer. These bonds would be less accessible to water and therefore less susceptible to hydrolysis. Of note, DSPC, which also contains ester bonds in the hydrophobic domain, also appears to be relatively stable within the limited context of this experiment. The three novel PEG-lipids, containing no ester bonds, were completely stable, as were the cholesterol and DODMA components.

To assess the utility of the PEG-lipids in formulations for systemic delivery, SPLP containing a luciferase reporter plasmid were administered to Neuro-2a tumor-bearing mice via tail vein injection. Since all the PEG-lipids used in this experiment were C$_{18}$ analogues, and the hydrophobic domain of the molecule is the dominant factor in determining transfection efficiency, it was not unexpected that the six formulations performed similarly. Each formulation resulted in comparable luciferase expression of approximately 700 pg/g of tumor. This compares favorably with previously reported SPLP formulations containing pCMVluc plasmids, which have yielded 30 [7] and 100 pg/g [9]. The preferential expression of luciferase in the tumor is in part due to passive disease site targeting and the EPR effect, but also because non-viral delivery systems transfect actively dividing cells more efficiently [45].

Analysis of serum transaminase levels confirmed comparable, low toxicity for all systems. AST and ALT levels were elevated only slightly in mice treated with 50 mg/kg total lipid, of which approximately 20 mg/kg was PEG-lipid, when compared to PBS controls. This was consistent with previous results in which SPLP have been shown to be less toxic than lipoplex systems [7]. The move from readily degradable ester PEG-lipids, to those with more stable, and possibly less easily metabolised bonds, had no apparent effect on acute toxicity.

The data favored the adoption of PEG-C-DSA or one of its analogues. PEG-C-DSA is stable in the bilayer at 40 °C for greater than six weeks, it is charge-neutral, formulates well, is synthesized in good yield with a minimum number of steps, and gives particles that transfect as well as PEG-S-DSG without signs of toxicity. To confirm the diffusible PEG-lipid paradigm would apply to PEG-C-DSA analogues and to address the issue of the immune response with more stably incorporated PEG-lipids, the C$_{14}$ analogue (PEG-C-DMA) of PEG-C-DSA was synthesized. The synthesis and purification of PEG-C-DMA, like its C$_{18}$ analogue, were found to be straightforward and of high yield (88% compared to 90%). PEG-C-DMA particles were found to possess the same small size and high encapsulation characteristics as those containing PEG-C-DSA (Table 1). The pharmacokinetics of PEG-C-DMA-containing SPLP was confirmed in PK studies utilizing ^{3}H-labelled SPLP. As with PEG-S-DSG/PEG-S-DMG formulations, where long ($t_{1/2}=15$ h) and short ($t_{1/2}=1$ h) circulating formulations were prepared by switching from the C$_{18}$ to a C$_{14}$ lipid anchor [9], the circulation half-life of PEG-C-DSA ($t_{1/2}=16$ h) and PEG-C-DMA ($t_{1/2}=2$ h) supported the diffusible PEG-lipid paradigm. Of interest, although the reduced circulation time had an effect on the resulting transfection efficiency (Fig. 7B), the tumor gene expression resulting from the administration of PEG-C-DMA-containing SPLP was as great as that obtained when using PEG-S-DSG suggesting that PEG-C-DMA may be a suitable replacement for PEG-S-DSG in nucleic acid delivery systems. Subsequently, toxicity evaluation by analysis of serum transferase levels was performed, following intravenous administration of PEG-C-DMA SPLP in A/J mice (data not shown). Similarly to the PEG-C-DSA analogue, AST and ALT levels of 125 and 78 IU/L, respectively, were barely elevated, and well within normal limits as set out by the Canadian Council on Animal Care (CCAC).

5. Conclusion

Using a simple assay developed to determine lipid degradation, we have demonstrated that two of the better-known types of PEG-lipid, PEG-S-DAGs and PEG-Ceramides, are unstable when incorporated in aqueous liposomal formulations. Accordingly, we have designed and synthesized three replacement PEG-lipids. We have shown that all three of the novel PEG-lipids are readily synthesized, stable under stressed conditions and formulate well as SPLP. The resulting particles are non-toxic and capable of transfecting distal tumors with 2 orders of magnitude of specificity over other organs. Our continuing changes to the lipid components of the SPLP particle, with no obvious penalty in terms of formulability or performance, further demonstrate the robustness of the SPLP platform and formulation methodology.
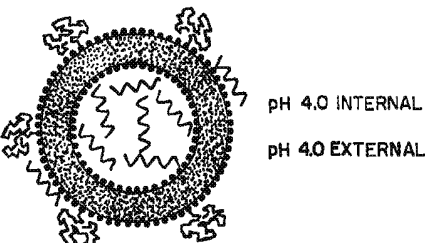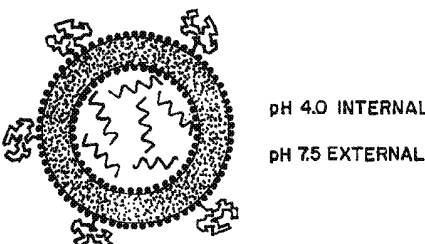
Acknowledgements

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JOINT APPENDIX 44

PCT

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(54) Title: HIGH EFFICIENCY ENCAPSULATION OF CHARGED THERAPEUTIC AGENTS IN LIPID VESICLES

(57) Abstract

Methods for the preparation of a lipid–nucleic acid composition are provided. According to the methods, a mixture of lipids containing a protonatable or deprotonatable lipid, for example an amino lipid and a lipid such as a PEG– or polyamide oligomer–modified lipid is combined with a buffered aqueous solution of a charged therapeutic agent, for example polyanionic nucleic acids, to produce particles in which the therapeutic agent is encapsulated in a lipid vesicle. Surface charges on the lipid particles are at least partially neutralized to provide surface–neutralized lipid–encapsulated compositions of the therapeutic agents. The method permits the preparation of compositions with high ratios of therapeutic agent to lipid and with encapsulation efficiencies in excess of 50 %.

pH 4.0 INTERNAL

pH 4.0 EXTERNAL

COLUMN CHROMATOGRAPHY

1. EXCHANGE pH 4.0 CITRATE FOR pH 7.5 HBS

2. NEUTRALIZE SURFACE DODAP; ANTISENSE RELEASE

3. REMOVAL OF NON-ENCAPSULATED ANTISENSE

RELEASE OF SURFACE ANTISENSE

pH 4.0 INTERNAL

pH 7.5 EXTERNAL

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CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
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CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
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DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

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HIGH EFFICIENCY ENCAPSULATION OF CHARGED
THERAPEUTIC AGENTS IN LIPID VESICLES

DESCRIPTION

FIELD OF THE INVENTION

This invention relates to compositions comprising a combination of a lipid and
a therapeutic agent, particularly to lipid-nucleic acid compositions, for *in vivo* therapeutic use.
In these compositions the therapeutic agent is encapsulated and protected from degradation

5   and clearance in serum. Additionally, the invention provides methods of making the
compositions, as well as methods of introducing the nucleic acids into cells using the
compositions and treating disease conditions.

BACKGROUND OF THE INVENTION

10   Therapeutic oligonucleotides, such as antisense oligonucleotides or ribozymes,
are short segments of DNA that have been designed to hybridize to a sequence on a specific
mRNA. The resulting complex can down-regulate protein production by several
mechanisms, including inhibition of mRNA translation into protein and/or by enhancement of
RNase H degradation of the mRNA transcripts. Consequently, therapeutic oligonucleotides

15   have tremendous potential for specificity of action (i.e. the down-regulation of a specific
disease-related protein). To date, these compounds have shown promise in several *in vitro*
and *in vivo* models, including models of inflammatory disease, cancer, and HIV (reviewed in
Agrawal, *Trends in Biotech.* 14:376-387 (1996)). Antisense can also effect cellular activity by
hybridizing specifically with chromosomal DNA.   Advanced human clinical assessments of

20   several antisense drugs are currently underway. Targets for these drugs include the genes or
RNA products of c-myc, ICAM-1, and infectious disease organisms such as cytomegalovirus,
and HIV-1.

One well known problem with the use of therapeutic oligonucleotides having a
phosphodiester internucleotide linkage is its very short half-life in the presence of serum or

25   within cells. (Zelphati, O et al. 1993. Inhibition of HIV-1 Replication in Cultured Cells with
Antisense Oligonucleotides Encapsulated in Immunoliposomes. Antisense. Res. Dev. 3:323-
338; and Thierry, AR et al. pp147-161 in Gene Regulation: Biology of Antisense RNA and

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DNA (Eds. Erickson, RP and Izant, JG) 1992. Raven Press, NY). No clinical assessment currently employs the basic phosphodiester chemistry found in natural nucleic acids, because of these and other known problems.

This problem has been partially overcome by chemical modifications which
5   reduce serum or intracellular degradation. Modifications have been tested at the internucleotide phosphodiester bridge (i.e. using phosphorthioate, methylphosphonate or phosphoramidate linkages), at the nucleotide base (i.e.5-propynyl-pyrimidines), or at the sugar (i.e. 2'-modified sugars) (Uhlmann E., et al. 1997. Antisense: Chemical Modifications. Encyclopedia of Cancer Vol. X. pp 64-81 Academic Press Inc.). Others have
10   attempted to improve stability using 2'-5' sugar linkages (see US Pat. No. 5,532,130). Other changes have been attempted. However, none of these solutions have proven entirely satisfactory, and *in vivo* free antisense still has only limited efficacy. Problems remain, such as in the limited ability of some antisense to cross cellular membranes (see, Vlassov, *et al., Biochim. Biophys. Acta* **1197**:95-1082 (1994)) and in the problems associated with systemic
15   toxicity, such as complement-mediated anaphylaxis, altered coagulatory properties, and cytopenia (Galbraith, *et al., Antisense Nucl. Acid Drug Des.* 4:201-206 (1994)). Further, as disclosed in US Pat. Appl. SN. 08/657,753 and counterpart patent application WO 97/46671, both incorporated herein by reference, modified antisense is still highly charged, and clearance from the circulation still takes place within minutes.

20   To attempt to improve efficacy, investigators have also employed lipid-based carrier systems to deliver chemically modified or unmodified antisense. In Zelphati, O and Szoka, F.C. (1996) J. Contr. Rel. 41:99-119, the authors refer to the use of anionic (conventional) liposomes, pH sensitive liposomes, immunoliposomes, fusogenic liposomes and cationic lipid/antisense aggregates.

25   None of these compositions successfully deliver phosphodiester antisense for *in vivo* therapy. In another paper, Zelphati & Szoka note that antisense phosphodiester oligonucleotides associated with cationic lipids have not been active in cell culture *in vitro*; and that only one study has reported the activity of phosphodiester antisense oligonucleotides complexed to cationic lipids. The authors argue that these findings "...necessitate[ ] the use
30   [of – sic] backbone-modified oligonucleotides that are relatively resistant to both intracellular and extracellular nucleases even if a carrier is used to deliver the oligonucleotide into the

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target cell". (1997. J. Lip. Res. 7(1):31-49 at 34). This finding is corroborated by Bennett, CF. (1995. Intracellular Delivery of Oligonucleotides with Cationic Liposomes. Chp 14 CRC Press) who states at p. 224 that "In contrast, we have been unable to demonstrate inhibition of gene expression by uniform phosphodiester oligodeoxynucleotides directed 5 towards a number of cellular targets in the presence of cationic lipids."

Prior art lipid formulations of modified antisense are also largely ineffective *in vivo*. They have poor encapsulation efficiency (15% or less for passive encapsulation systems), poor drug to lipid ratios (3% or less by weight), high susceptibility to serum nucleases and rapid clearance from circulation (particularly in the case of cationic 10 lipid/antisense aggregates made from DOTMA, trade-name LIPOFECTIN™), and/or large sized particles (greater than 100 nm), which make them unsuitable for systemic delivery to target sites. No successful *in vivo* efficacy studies of lipid-encapsulated (nuclease-resistant) modified antisense are known in the prior art.

Two references to unique lipid-antisense compositions that may be 15 significantly nuclease resistant bear consideration. Firstly, the anionic liposome (LPDII) composition of Li, S. and Huang, L (1997. J. Lip. Res. 7(1) 63-75), which encapsulates poly-lysine coated antisense, are said to have 60-70% encapsulation efficiency, but suffer from a large size of around 200 nm and a low drug to lipid ratio of 8% by weight. The effect of these particles *in vivo* is unknown. Secondly, the Minimal Volume Entrappment (MVE) 20 technique for cardiolipin (anionic) liposomes results in the reasonably high encapsulation efficiency of 45-65% but again the drug:lipid ratio remains very small, approximately 6.5% by weight (see US Pat. No. 5,665,710 to Rahman et al.; Thierry AR, and Takle, GB. 1995, Liposomes as a Delivery System for Antisense and Ribozyme Compounds. in *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, S. Akhtar, ed, CRC Press, Boca 25 Raton, FL., pp. 199-221; Thierry, AR et al. pp147-161 in Gene Regulation: Biology of Antisense RNA and DNA (Eds. Erickson, RP and Izant, JG) 1992. Raven Press, NY). Note that US Pat. No. 5,665,710 also discloses encapsulation efficiencies of 60-90% for tiny, medically useless amounts of antisense (0.1 ug), where the drug to lipid ratio must be very low.

30   It is an observation of the inventors that a wide variety of prior art lipid compositions used for conventional drugs could be tested for efficacy in the antisense field,

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but the improvement (over free antisense) for *in vivo* efficacy is not known. In this regard, it is noted that although lipid compositions assertedly for use as drug carriers were disclosed by Bailey and Cullis (US Pat. 5552155; and (1994) Biochem. 33(42):12573-12580), they did not disclose formulations of any bioactive compounds with these lipids, and did not suggest their
5   utility for high efficiency loading of polyanionic species.

What is needed in the art are improved lipid-therapeutic oligonucleotide compositions which are suitable for therapeutic use. Preferably these compositions would encapsulate nucleic acids with high-efficiency, have high drug:lipid ratios, be encapsulated and protected from degradation and clearance in serum, and/or be suitable for systemic
10   delivery. The present invention provides such compositions, methods of making the compositions and methods of introducing nucleic acids into cells using the compositions and methods of treating diseases.

SUMMARY OF THE INVENTION

15   In accordance with the invention, charged therapeutic agents are packaged into lipid-encapsulated therapeutic agent particles using a method comprising the steps of:

(a)   combining a mixture of lipids comprising at least a first lipid component and a second lipid component with a buffered aqueous solution of a charged therapeutic agent to form an intermediate mixture containing lipid-encapsulated therapeutic
20   particles, and

(b) changing the pH of the intermediate mixture to neutralize at least some exterior surface charges on said lipid-nucleic acid particles to provide at least partially-surface neutralized lipid-encapsulated therapeutic agent particles. The first lipid component is selected from among lipids containing a protonatable or deprotonatable group that has a
25   pKa such that the lipid is in a charged form at a first pH and a neutral form at a second pH, preferably near physiological pH. The buffered solution has a pH such that the first lipid component is in its charged form when in the buffered solution, and the first lipid component is further selected such that the charged form is cationic when the therapeutic agent is anionic in the buffered solution and anionic when the therapeutic agent is cationic in the buffered
30   solution. The second lipid component being selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation. The method the invention is

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particularly useful for preparation of lipid-encapsulated nucleic acids, for example antisense nucleic acids or ribozyme.

BRIEF DESCRIPTION OF THE DRAWINGS

5   **Figure 1** illustrates a neutralization step which releases surface-bound antisense from the lipid-nucleic acid compositions according to the present invention.

Figures 2A and 2B illustrate certain lipid components which are useful in the present inventive methods. Figure 2A illustrates several groups of amino lipids including the chemical structure of DODAP. Figure 2B illustrates groups of PEG-modified lipids.

10   **Figure 3** illustrates the influence of ethanol on the encapsulation of antisense oligodeoxynucleotides. The liposomal antisense compositions were prepared as described in the Examples, with the final concentrations of antisense and lipids being 2 mg/mL and 9.9 mg/mL, respectively. The final ethanol concentration in the preparations was varied between 0 and 60%, vol/vol. Encapsulation was assessed either by analyzing the pre-column and

15   post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

Figure 4 illustrates the influence of ethanol on lipid and antisense loss during extrusion. The liposomal antisense compositions were prepared as described for Figure 3. The samples were extruded ten times through three 100 nm filters as described in "Materials

20   and Methods". After extrusion, the filters were analyzed for [^{3}H]-antisense and [^{14}C]-lipid radioactivity by standard scintillation counting techniques. Results were expressed as a percent of the total initial radioactivity.

Figure 5 illustrates the influence of DODAP content on the encapsulation of antisense oligodeoxynucleotides. A 0.6 mL aliquot of a [^{3}H]-phosphorothioate antisense

25   oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with 0.4 mL of a 95% ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 100-(55+X):45:X:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The molar ratio of DODAP was varied between 0 and 30%. The molar ratio of DSPC was adjusted to compensate for the changes in DODAP content. Encapsulation was assessed either by

30   analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by

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determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

       Figure 6 illustrates the influence of DODAP content on the encapsulation of antisense oligodeoxynucleotides. Samples were identical to those prepared in Figure 5. In

5   this instance, the amount of antisense associated with the lipid was assessed by a solvent extraction procedure as described in "Material and Methods". Antisense was extracted into a methanol:water aqueous phase, while the lipid was soluble in the organic (chloroform) phase. The aqueous phase was preserved and antisense concentration was determined by measuring the absorbance at 260 nm. This confirmed that the antisense was associated with the lipid

10   vesicles, and that the [^{3}H]-label on the antisense had not exchanged to the lipid.

       Figure 7 illustrates the quasi-elastic light scattering analysis of encapsulated liposomal antisense. The size distribution of a liposomal preparation of antisense was determined by quasi-elastic light scattering (QELS) immediately after removal of the free antisense (A), and after storage of the preparation for 2 months at 4°C (B), using a Nicomp

15   Model 370 sub-micron particle sizer.

       Figure 8 illustrates the influence of the initial antisense concentration on antisense loading in DODAP vesicles. Varying final concentrations of a 20mer of [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) were mixed with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14;

20   25:45:20:10, molar ratio), 9.9 mg/mL (final concentration). Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity. EPC:CHOL liposomes containing encapsulated antisense are included for comparison.

25   **Figure 9** illustrates the plasma clearance of encapsulated antisense. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP:PEG-CerC14 (25:45:20:10), where X represents either distearoylphosphatidylcholine (DSPC),

30   sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected

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(200 µL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

Figure 10 illustrates the biodistribution of encapsulated antisense.

5   Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP: PEG-CerC14 (25:45:20:10), where X represents either distearoyl-phosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The

10   formulations contained a lipid label ($[^{14}C]$-cholesterylhexadecylether) and $[^3H]$-antisense and were injected (200 µL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Mice were terminated by cervical dislocation and the organs were recovered and processed as described in "Materials and Methods". Lipid and antisense recoveries were determined by standard scintillation counting techniques.

15   **Figure 11** illustrates the differential release rates of antisense in plasma. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP: PEG-CerC14 (25:45:20:10), where X represents either distearoyl-phosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The

20

formulations contained a lipid label ($[^{14}C]$-cholesterylhexadecylether) and $[^3H]$-antisense and were injected (200 µL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

25   Release rates were determined by measuring the [3H]/[14C] ratio over time.

Figure 12 illustrates the influence of PEG-acyl chain lengths on plasma clearance of encapsulated antisense. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were

30   composed of DSPC:CHOL:DODAP:PEG-CerC14 or C20 (25:45:20:10). The formulation contained a lipid label ($[^{14}C]$-cholesterylhexadecylether) and $[^3H]$-antisense and were injected

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(200 µL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

5   **Figure 13** illustrates the enhanced efficacy of liposomal antisense containing DODAP - ear swelling. Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped PS 3082 (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped PS 3082 (identified as 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped PS 3082 (identified as 4200). Ear swelling was measured at 24 hours after initiating inflammation 10   using an engineer's micrometer.

Figure 14 illustrates the enhanced efficacy of liposomal antisense containing DODAP - cellular infiltration. Mice received 10 µCi of [^{3}H]-methylthymidine, i.p., 24 hours before initiating inflammation. Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped PS 3082 15   (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped PS 3082 (identified as 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped PS 3082 (identified as 4200). Cell infiltration was monitored by measuring the radioactivity in the "challenged ear" versus the non-treated ear. Results are expressed as the ratio of radioactivity in the left (challenged ear) versus right ear.

20   **Figure 15** shows asymmetric loading of lipid-encapsulated-nucleic acid particles in accordance with the invention .

Figure 16 shows clearance of lipid-encapsulated antisense particles formulated with several amino lipids at different levels.

Figure 17 shows blood levels of antisense-containing particles after repeat 25   dosages.

Figure 18 shows blood levels of antisense-containing particles after repeat dosages.

Figure 19 illustrates results of a study on the *in vivo* efficacy of lipid-encapsulated antisense particles in accordance with the invention in a mouse tumor model.

30   **Figure 20** shows encapsulation efficiency results for lipid-encapsulated therapeutic agent particles in accordance with the invention.

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Figure 21 shows results for studies on the use of murine ICAM1 in an ear inflammation model.

Figure 22 shows results for studies on the use of murine ICAM1 in an ear inflammation model.

5   **Figure 23** shows results for studies on the use of murine ICAM1 in an ear inflammation model.

DETAILED DESCRIPTION OF THE INVENTION

CONTENTS

I.   Glossary

Abbreviations and Definitions

20   The following abbreviations are used herein: ATTA, N-(ω-N'-acetoxy-octa(14'amino-3',6',9',12'-tetraoxatetradecanoyl)); CHE, cholesteryl-hexadecylether; CHOL, cholesterol; DODAP or AL-1, 1,2-dioleoyloxy-3-dimethylaminopropane (and its pro-tonated ammonium form); DODMA, N-(1-(2,3-Dioleoyloxy) propyl)-N,N,-dimethyl ammonium chloride; DSPC, distearoylphosphatidylcholine; EPC, egg phosphatidylcholine; 25   HBS, HEPES-buffered saline; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; MES, 2-(N-morpholino)ethane sulfonic acid; PS 3082, murine ICAM-1 phosphoro-thioate oligodeoxynucleotide having the sequence: TGCATCCCCCAGGCCACCAT (SEQ ID No. 1); NaCl, sodium chloride; OLIGREEN™, a dye that becomes fluorescent when interacting with an oligonucleotide; PEG-CerC20, polyethylene glycol coupled to a ceramide 30   derivative with 20 carbon acyl chain; POPC, palmitoyloleoylphophatidylcholine; SM, sphingomyelin.

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"Lipid-therapeutic agent particle" means a particle comprising lipids and a charged (cationic or anionic) therapeutic agent.   "Lipid-therapeutic nucleic acid particle" means a particle comprising a lipid and a therapeutic nucleic acid.

"Lipid-encapsulated therapeutic agent (nucleic acid) particle" means a lipid-
5   therapeutic agent particle wherein less than 50% and preferably less than 10% of the therapeutic agent (nucleic acid) is detectable on the external surface of the particle or in the buffer external to the particle. In the case of nucleic acids, the amount of encapsulated versus unencapsulated nucleic acid can be assayed by fluorescence assays or nuclease assays as described herein. Comparable assays can be used for other types of therapeutic agents.

10   "Therapeutically effective amount" means an amount which provides a therapeutic benefit.   For antisense oligonucleotide this means generally 0.5 to 50 mg/kg of body weight, but when delivered in a lipid particle formulation, a below-toxic amount of lipid must be used.

"Lipid exchange out of particle" and the rate of this exchange is fully
15   explained in US Pat. Apps. SN 08/486,214 and 08/485,608 and PCT Patent publications WO 96/10391 and WO 96/10392, which are all incorporated herein by reference. Lipid exchange into the surrounding medium is possible for lipids which are reversibly associated with the lipid particle membrane. Each lipid has a characteristic rate at which it will exchange put of a particle which depends on a variety of factors including acyl chain length, saturation, head
20   group size, buffer composition and membrane composition.

"Disease site" is the site in an organism which demonstrates or is the source of a pathology. The disease site may be focused, as in a site of neoplasm or inflammation, or may be diffuse as in the case of a non-solid tumor. "Administration at a site which is distal to the disease site" means that delivery to the disease site will require some kind of systemic
25   delivery, either by blood or lymph circulation, or other fluid movement inside the organism.

The term "transfection" as used herein, refers to the introduction of polyanionic materials, particularly nucleic acids, into cells. The polyanionic materials can be in the form of DNA or RNA which is linked to expression vectors to facilitate gene expression after entry into the cell. Thus the polyanionic material or nucleic acids used in the
30   present invention is meant to include DNA having coding sequences for structural proteins, receptors and hormones, as well as transcriptional and translational regulatory elements (*i.e.,*

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promoters, enhancers, terminators and signal sequences) and vector sequences. Methods of
incorporating particular nucleic acids into expression vectors are well known to those of skill
in the art, but are described in detail in, for example, Sambrook *et al.*, *Molecular Cloning: A
Laboratory Manual* (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) or *Current*
5   *Protocols in Molecular Biology*, F. Ausubel *et al.*, ed. Greene Publishing and Wiley-
Interscience, New York (1987), both of which are incorporated herein by reference.

The term "physiological pH" refers to pH levels conventionally encountered in
serum or blood. In general, this will be in the range of pH 7.2 to 7.5. Preferred protonatable
or deprotonatable lipids have a pKa such that they are substantially neutral at this pH, i.e., a
10   pKa of about 4 to 7 in the case of an amino lipid.

II.   General

The present invention relates to methods and compositions for producing
lipid-encapsulated therapeutic agent particles in which charged therapeutic agents are
15   encapsulated within a lipid layer. The invention is applicable to both anionic and cationic
therapeutic agents, including polyanionic nucleic acids, polyanionic proteins or peptides,
cytokines and heparin, and cationic proteins and peptides. The invention is principally
demonstrated herein with reference to polyanionic nucleic acids as the therapeutic agent,
which is a preferred embodiment, but the same principles can be readily extended to other
20   polyanionic or to cationic therapeutic agents.

To evaluate the quality of a lipid/nucleic acid formulation the following
criteria, among others, may be employed:

drug to lipid ratio;

encapsulation efficiency;

25   nuclease resistance/serum stability; and

particle size.

High drug to lipid rations, high encapsulation efficiciency, good nuclease resistance and
serum stability and controllable particle size, generally less than 200 nm in diameter are
desirable In addition, the nature of the nucleic acid polymer is of significance, since the
30   modification of nucleic acids in an effort to impart nuclease resistance adds to the cost of
therapeutics while in many cases providing only limited resistance. The present invention

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provides lipid-nucleic acid particles and methods for preparing lipid-nucleic acid

formulations which are far superior to the art according to these criteria.

Unless stated otherwise, these criteria are calculated in this specification as

follows:

5   drug to lipid ratio: The amount of drug (therapeutic agent) in a defined

volume of preparation divided by the amount of lipid in the same volume. This may be on a

mole per mole basis or on a weight per weight basis, or on a weight per mole basis. For final,

administration-ready formulations, the drug:lipid ratio is calculated after dialysis,

chromatography and/or enzyme (e.g., nuclease) digestion has been employed to remove as

10   much of the external therapeutic agent (e.g., nucleic acid) as possible. Drug:lipid ratio is a

measure of potency of the formulation, although the highest possible drug:lipid ratio is not

always the most potent formulation;

encapsulation efficiency: the drug to lipid ratio of the starting mixture divided

by the drug to lipid ratio of the final, administration competent formulation. This is a

15   measure of relative efficiency. For a measure of absolute efficiency, the total amount of

therapeutic agent (nucleic acid) added to the starting mixture that ends up in the

administration competent formulation, can also be calculated. The amount of lipid lost

during the formulation process may also be calculated. Efficiency is a measure of the

wastage and expense of the formulation;

20   nuclease resistance/serum stability: the ability of the formulation to protect

the nucleic acid therapeutic agents from nuclease digestion either in an *in vitro* assay, or in

circulation. Several standard assays are detailed in this specification. Encapsulated particles

have much greater nuclease resistance and serum stability than lipid-antisense aggregates

such as DOTMA/DOPE (LIPOFECTIN™) formulations; and

25   size: the size of the particles formed. Size distribution may be determined

using quasi-elastic light scattering (QELS) on a Nicomp Model 370 sub-micron particle sizer.

Particles under 200 nm are preferred for distribution to neo-vascularized (leaky) tissues, such

as neoplasms and sites of inflammation.

The methods and composition of the invention make use of certain lipids

30   which can be present in both a charged and an uncharged form. For example, amino lipids

which are charged at a pH below the pK_a of the amino group and substantially neutral at a pH

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above the pK$_a$ can be used in a two-step process. First, lipid vesicles can be formed at the lower pH with (cationic) amino lipids and other vesicle components in the presence of nucleic acids. In this manner the vesicles will encapsulate and entrap the nucleic acids. Second, the surface charge of the newly formed vesicles can be neutralized by increasing the pH of the

5 medium to a level above the pK$_a$ of the amino lipids present, i.e., to physiological pH or higher. Particularly advantageous aspects of this process include both the facile removal of any surface adsorbed nucleic acid and a resultant nucleic acid delivery vehicle which has a neutral surface. Liposomes or lipid particles having a neutral surface are expected to avoid rapid clearance from circulation and to avoid certain toxicities which are associated with

10 cationic liposome preparations.

It is further noted that the vesicles formed in this manner provide formulations of uniform vesicle size with high content of nucleic acids. Additionally, the vesicles are not aggregate complexes, but rather are large unilamellar vesicles having a size range of from about 70 to about 200 nm, more preferably about 90 to about 130 nm.

15 Without intending to be bound by any particular theory, it is believed that the very high efficiency of nucleic acid encapsulation is a result of electrostatic interaction at low pH. Figure 1 provides an illustration of the processes described herein. More particularly, this figure illustrates a lipid-nucleic acid composition of amino lipids and PEG-modified lipids having encapsulated antisense nucleic acid and surface-bound antisense nucleic acid.

20 At acidic pH (shown as pH 4.0), the surface is charged and binds a portion of the antisense through electrostatic interactions. When the external acidic buffer is exchanged for a more neutral (pH 7.5, HBS) buffer, the surface of the lipid particle or liposome is neutralized, resulting in release of the antisense nucleic acid.

Encapsulation efficiency results in Figs. 15 show a further unexpected benefit

25 of the invention. As shown in the figure, for both phosphorothioate (PS-2302) and phosphodiester (PO-2302) formulations it is possible to obtain encapsulation efficiencies – i.e., the amount of nucleic acid that ends up on the inside of the particle – that are greater than 50%. Phosphodiesters achieve well over 60%, and phosphorothioates can be at least up to 80% encapsulated. The assymetry of loading is surprising, given that in the simplest model

30 of loading large unilamellar vesicles (LUV's) the therapeutic agent (nucleic acid) would be equally likely to associate with cationic charges on the inside and outside of the particle. A

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1:1 distribution (inside to outside) would suggest that the 50% on the outside should be removed upon neutralization of the outside surface charges, such that 50% efficiency would be the theoretical upper limit.   Through some unclear mechanism, however, the invention surprisingly provides an active process whereby the majority of the therapeutic agent (nucleic

5   acid) ends up protected on the inside of the particles.

III.   Methods of Preparing Lipid/Therapeutic Agent (Nucleic Acid) Formulations

In view of the above, the present invention provides methods of preparing lipid/nucleic acid formulations. In the methods described herein, a mixture of lipids is

10   combined with a buffered aqueous solution of nucleic acid to produce an intermediate mixture containing nucleic acid encapsulated in lipid particles wherein the encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 10 wt% to about 20 wt%. The intermediate mixture may optionally be sized to obtain lipid-encapsulated nucleic acid particles wherein the lipid portions are large unilamellar vesicles, preferably having a

15   diameter of 70 to 200 nm, more preferably about 90 to 130 nm.   The pH is then raised to neutralize at least a portion of the surface charges on the lipid-nucleic acid particles, thus providing an at least partially surface-neutralized lipid-encapsulated nucleic acid composition.

The mixture of lipids includes at least two lipid components: a first lipid

20   component that is selected from among lipids which have a pKa such that the lipid is cationic at pH below the pKa and neutral at pH above the pKa, and a second lipid component that is selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation.

The first lipid component of is a lipid (or a mixture of lipid species with

25   similar properties) which has at least one protonatable or deprotonatable group, such that the lipid is charged at a first pH (cationic or anionic, depending on the nature and pKa of the protonatable or deprotonatable group), and neutral at physiological pH. It will of course be understood that the addition or removal of protons as a function of pH is an equilibrium process, and that the reference to a charged or a neutral lipid refers to the nature of the

30   predominant species and does not require that all of the lipid be present in the charged or neutral form.   Lipids which have more than one protonatable or deprotonatable group, or
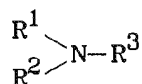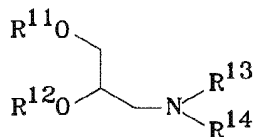
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which are zwitterionic are not excluded from use in the invention. Protonatable lipids are particularly useful as the first lipid component of the invention when the pKa of the protonatable group is in the range of about 4 to about 11. Most preferred is pKa of about 4 to about 7, because these lipids will be cationic at the lower pH formulation stage, while

5   particles will be largely (though not completely) surface neutralized at physiological pH around pH 7.5. One of the benefits of this pKa is that at least some antisense stuck to the outside surface of the particle will lose its electrostatic interaction at physiological pH and be removed by simple dialysis; thus greatly reducing the particle's susceptibility to clearance.

Preferred lipids with a protonatable group for use as the first lipid component

10   of the lipid mixture are amino lipids. As used herein, the term "amino lipid" is meant to include those lipids having one or two fatty acid or fatty alkyl chains and an amino head group (including an alkylamino or dialkylamino group) which is protonated to form a cationic lipid at physiological pH (see Figure 2A). In one group of embodiments, the amino lipid is a primary, secondary or tertiary amine represented by the formula:

15

$$\begin{array}{c} R^1 \\ \diagdown \\ R^2 \diagup N{-}R^3 \end{array}$$

in which R^1 is a C_{12} to C_{24} alkyl group which is branched or unbranched, and saturated or unsaturated. R^2 is hydrogen or a C_1 to C_{24} alkyl group which is also branched or unbranched, and saturated or unsaturated (when three or more carbons are present). R^3 is hydrogen or a C_1 to C_6 alkyl group. Examples of these amino lipids include, for example, stearylamine,

20   oleylamine, dioleylamine, N-methyl-N,N-dioleylamine, and N,N-dimethyloleylamine.

In another group of embodiments, the amino lipid is a lipid in which the amino head group is attached to one or more fatty acid or fatty alkyl groups by a scaffold such as, for example, a glycerol or propanediol moiety. Illustrative of these amine lipids is the formula:

25

$$\begin{array}{c} R^{11}O \\ \diagdown \\ \diagup \diagdown \\ R^{12}O \qquad \diagup N \diagup R^{13} \\ \diagdown R^{14} \end{array}$$

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wherein at least one and preferably both of R^{11} and R^{12} is a C_{12} to C_{24} alkyl or acyl group which is branched or unbranched, saturated or unsaturated. In those embodiments in which only one of R^{11} or R^{12} is a long chain alkyl or acyl group, the other of R^{11} or R^{12} will be a hydrogen or lower alkyl or acyl group having from one to six carbon atoms. The remaining

5   groups, R^{13} and R^{14} are typically hydrogen or C_1 to C_4 alkyl. In this group of embodiments, the amino lipid can be viewed as a derivative of 3-monoalkyl or dialkylamino-1,2-propanediol. An example of a suitable amino lipid is DODAP (1,2-dioleoyloxy-3-dimethylamino-propane, see Figure 2A). Other amino lipids would include those having alternative fatty acid groups and other dialkylamino groups, including

10   those in which the alkyl substitutents are different (*e.g.,* N-ethyl-N-methylamino-, N-propyl-N-ethylamino- and the like). For those embodiments in which R^{11} and R^{12} are both long chain alkyl or acyl groups, they can be the same or different. In general, amino lipids having less saturated acyl chains are more easily sized, particularly when the complexes must be sized below about 0.3 microns, for purposes of filter sterilization. Amino lipids containing

15   unsaturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are particularly preferred. Other scaffolds can also be used to separate the amino group and the fatty acid or fatty alkyl portion of the amino lipid. Suitable scaffolds are known to those of skill in the art.

Compounds that are related to DODAP that may be useful with this invention include: 1-oleoyl-2-hydroxy-3-N,N-dimethylamino propane; 1,2-diacyl-3-N,N-

20   dimethylamino propane; and 1,2-didecanoyl-1-N,N-dimethylamino propane. Further, it is proposed that various modifications of the DODAP or DODMA headgroup, or any compound of the general formula:

can be modified to obtain a suitable pKa. Suitable headgroup modifications that are useful in the instant invention include:

25

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	R^1	R^2
1	H	H
2	H	CH_3
3	CH_3	CH_3
4	H	CH_2CH_3
5	CH_3	CH_2CH_3
6	CH_2CH_3	CH_2CH_3
7	H	CH_2CH_2OH
8	CH_3	CH_2CH_2OH
9	CH_2CH_3	CH_2CH_2OH
10	CH_2CH_2OH	CH_2CH_2OH
11*	H	$CH_2CH_2NH_2$
12*	CH_3	$CH_2CH_2NH_2$
13*	CH_2CH_3	$CH_2CH_2NH_2$
14*	CH_2CH_2OH	$CH_2CH_2NH_2$
15*	$CH_2CH_2NH_2$	$CH_2CH_2NH_2$

In other embodiments, the amino lipid can be a derivative of a naturally occurring amino lipid, for example, sphingosine. Suitable derivatives of sphingosine would include those having additional fatty acid chains attached to either of the pendent hydroxyl groups, as well as alkyl groups, preferably lower alkyl groups, attached to the amino functional group.

Other lipids which may be used as the first lipid component of the invention include phosphine lipids (although toxicity issues may limit their utility), and carboxylic acid lipid derivative. These generally have a pKa of about 5 and are therefore useful with cationic therapeutic agents.

The second lipid component is selected to improve the formulation process by reducing aggregation of the lipid particles during formation. This may result from steric stabilization of particles which prevents charge-induced aggregation during formation. Examples of suitable lipids for this purpose include polyethylene glycol (PEG)-modified lipids, monosialoganglioside Gm1, and polyamide oligomers ("PAO") such as ATTA

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(disclosed in US Pat. Appl. SN 60/073,852 and US Pat. Appl. SN 60/(not yet received TT&C Attorney Docket No.16303-005810 both assigned to the assignee of the instant invention and incorporated herein by reference). Other compounds with uncharged, hydrophilic, steric-barrier moieties, that prevent aggregation during formulation, like PEG, Gm1 or ATTA, can

5   also be coupled to lipids for use as the second lipid component in the methods and compositions of the invention. Typically, the concentration of the second lipid component is about 1 to 15% (by mole percent of lipids).

Specific examples of PEG-modified lipids (or lipid-polyoxyethylene conjugates) that are useful in the present invention can have a variety of "anchoring" lipid

10   portions to secure the PEG portion to the surface of the lipid vesicle. Examples of suitable PEG-modified lipids include PEG-modified phosphatidylethanolamine and phosphatidic acid (see Figure 2B, structures A and B), PEG-modified diacylglycerols and dialkylglycerols (see Figure 2B, structures C and D), PEG-modified dialkylamines (Figure 2B, structure E) and PEG-modified 1,2-diacyloxypropan-3-amines (Figure 2B, structure F). Particularly preferred

15   are PEG-ceramide conjugates (e.g., PEG-CerC14 or PEG-CerC20) which are described in co-pending USSN 08/486,214, incorporated herein by reference.

In embodiments where a sterically-large moiety such as PEG or ATTA are conjugated to a lipid anchor, the selection of the lipid anchor depends on what type of association the conjugate is to have with the lipid particle. It is well known that

20   mePEG(mw2000)-diastearoyl-phosphatidylethanolamine (PEG-DSPE) will remain associated with a liposome until the particle is cleared from the circulation, possibly a matter of days. Other conjugates, such as PEG-CerC20 have similar staying capacity. PEG-CerC14, however, rapidly exchanges out of the formulation upon exposure to serum, with a $T_{1/2}$ less than 60 mins. in some assays. As illustrated in US Pat. Application SN 08/486,214

25   at least three characteristics influence the rate of exchange: length of acyl chain, saturation of acyl chain, and size of the steric-barrier head group. Compounds having suitable variations of these features may be useful for the invention.

In addition to the first and second lipid components, the lipid mixture may contain additional lipid species. These additional lipids may be, for example, neutral lipids or

30   sterols.

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Neutral lipids, when present in the lipid mixture, can be any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, for example diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, and cerebrosides. The

5   selection of neutral lipids for use in the complexes herein is generally guided by consideration of, *e.g.*, liposome size and stability of the liposomes in the bloodstream. Preferably, the neutral lipid component is a lipid having two acyl groups, (*i.e.*, diacylphosphatidylcholine and diacylphosphatidylethanolamine). Lipids having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be

10   isolated or synthesized by well-known techniques. In one group of embodiments, lipids containing saturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are preferred. In another group of embodiments, lipids with mono or diunsaturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are used. Additionally, lipids having mixtures of saturated and unsaturated fatty acid chains can be used. Preferably, the neutral

15   lipids used in the present invention are DOPE, DSPC, POPC, or any related phosphatidylcholine. The neutral lipids useful in the present invention may also be composed of sphingomyelin or phospholipids with other head groups, such as serine and inositol.

The sterol component of the lipid mixture, when present, can be any of those sterols conventionally used in the field of liposome, lipid vesicle or lipid particle preparation.

20   A preferred sterol is cholesterol.

The mixture of lipids is typically a solution of lipids in an alcoholic solvent. Hydrophilic, low molecular weight water miscible alcohols with less than 10 carbon atoms, preferably less than 6 carbon atoms are preferred. Typical alcohols used in this invention are ethanol, methanol, propanol, butanol, pentanol and ethylene glycol and propylene glycol.

25   Particularly preferred is ethanol. In most embodiments, the alcohol is used in the form in which it is commercially available. For example, ethanol can be used as absolute ethanol (100%), or as 95% ethanol, the remainder being water.

In one exemplary embodiment, the mixture of lipids is a mixture of amino lipids, neutral lipids (other than an amino lipid), a sterol (*e.g.*, cholesterol) and a PEG-

30   modified lipid (*e.g.*, a PEG-ceramide) in an alcohol solvent. In preferred embodiments, the lipid mixture consists essentially of an amino lipid, a neutral lipid, cholesterol and a PEG-

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ceramide in alcohol, more preferably ethanol. In further preferred embodiments, the first

solution consists of the above lipid mixture in molar ratios of about 10-35% amino lipid:25-

45% neutral lipid:35-55% cholesterol:0.5-15% PEG-ceramide. In still further preferred

embodiments, the first solution consists essentially of DODAP, DSPC, Chol and PEG-

5   CerC14, more preferably in a molar ratio of about 10-35% DODAP:25-45% DSPC:35-55%

Chol:0.5-15% PEG-CerC14. In another group of preferred embodiments, the neutral lipid in

these compositions is replaced with POPC or SM.

In accordance with the invention, the lipid mixture is combined with a

buffered aqueous solution of charged therapeutic agent, preferably nucleic acids. The

10   buffered aqueous solution of therapeutic agents (nucleic acids) which is combined with the

lipid mixture is typically a solution in which the buffer has a pH of less than the pK_a of the

protonatable lipid in the lipid mixture. As used herein, the term "nucleic acid" is meant to

include any oligonucleotide or polynucleotide having from 10 to 100,000 nucleotide residues.

Antisense and ribozyme oligonucleotides are particularly preferred. The term "antisense

15   oligonucleotide" or simply "antisense" is meant to include oligonucleotides which are

complementary to a targeted nucleic acid and which contain from about 10 to about 50

nucleotides, more preferably about 15 to about 30 nucleotides. The term also encompasses

antisense sequences which may not be exactly complementary to the desired target gene.

Thus, the invention can be utilized in instances where non-target specific-activities are found

20   with antisense, or where an antisense sequence containing one or more mismatches with the

target sequence is the most preferred for a particular use.

The nucleic acid that is used in a lipid-nucleic acid particle according to this

invention includes any form of nucleic acid that is known. Thus, the nucleic acid may be a

modified nucleic acid of the type used previously to enhance nuclease resistance and serum

25   stability. Surprisingly, however, acceptable therapeutic products can also be prepared using

the method of the invention to formulate lipid-nucleic acid particles from nucleic acids which

have no modification to the phosphodiester linkages of natural nucleic acid polymers, and the

use of unmodified phosphodiester nucleic acids (i.e., nucleic acids in which all of the linkages

are phosphodiester linkages) is a preferred embodiment of the invention. Still other nucleic

30   acids which are useful in the present invention include, synthetic or pre-formed poly-RNA

such as poly(IC) IC.

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The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or DNA-RNA hybrids. Examples of double-stranded DNA include structural genes, genes including control and termination regions, and self-replicating systems such as plasmid DNA. Single-stranded nucleic acids include antisense oligonucleotides (discussed
5   above and complementary to DNA and RNA), ribozymes and triplex-forming oligonucleotides.

In order to increase stability, some single-stranded nucleic acids may have some or all of the nucleotide linkages substituted with stable, non-phosphodiester linkages, including, for example, phosphorothioate, phosphorodithioate, phosphoroselenate,
10   boranophosphate, methylphosphonate, or O-alkyl phosphotriester linkages. Phosphorothioate nucleic acids (PS-oligos) are those oligonucleotides or polynucleotides in which one of the non-bridged oxygens of the internucleotide linkage has been replaced with sulfur. These PS-oligos are resistant to nuclease degradation, yet retain sequence-specific activity. Similarly, phosphorodithioate nucleic acids are those oligonucleotides or
15   polynucleotides in which each of the non-bridged oxygens of the internucleotide linkage have been replaced by a sulfur atom. These phosphorodithioate-oligos have also proven to be more nuclease resistant than the natural phosphodiester-linked form. Other useful nucleic acids derivatives include those nucleic acids molecules in which the bridging oxygen atoms (those forming the phosphoester linkages) have been replaced with $-S-$, $-NH-$, $-CH_2-$ and the
20   like. Preferably, the alterations to the antisense or other nucleic acids used will not completely affect the negative charges associated with the nucleic acids. Thus, the present invention contemplates the use of antisense and other nucleic acids in which a portion of the linkages are replaced with, for example, the neutral methyl phosphonate or phosphoramidate linkages. When neutral linkages are used, preferably less than 80% of the nucleic acid
25   linkages are so substituted, more preferably less than 50%.

Those skilled in the art will realize that for *in vivo* utility, such as therapeutic efficacy, a reasonable rule of thumb is that if a thioated version of the sequence works in the free form, that encapsulated particles of the same sequence, of any chemistry, will also be efficacious. Encapsulated particles may also have a broader range of *in vivo* utilities,
30   showing efficacy in conditions and models not known to be otherwise responsive to antisense therapy. Those skilled in the art know that applying this invention they may find old models

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which now respond to antisense therapy. Further, they may revisit discarded antisense

sequences or chemistries and find efficacy by employing the invention.

Therapeutic antisense sequences (putatatively target specific) known to work with this

invention include the following:

5

Trivial Name:	Gene Target, Chemistry and Sequence	
PS-3082	murine ICAM-1 (Intracellular Adhesion Molecule-1) (phosphorothioate)	
	TGCATCCCCCAGGCCACCAT	(SEQ ID. No 1)
PO-3082	murine ICAM-1 (phosphodiester)	
	TGCATCCCCCAGGCCACCAT	(SEQ ID. No 1)
PS-2302	human ICAM-1 (phosphorothioate)	
	GCCCAAGCTGGCATCCGTCA	(SEQ ID. No 2)
PO-2302	human ICAM-1 (phosphodiester)	
	GCCCAAGCTGGCATCCGTCA	(SEQ ID. No 2)
PS-8997	human ICAM-1 (phosphorothioate)	
	GCCCAAGCTGGCATCCGTCA	(SEQ ID. No 2)
PO- 8997	human ICAM-1 (phosphodiester)	
	GCCCAAGCTGGCATCCGTCA	(SEQ ID. No 2)
US3	human erb-B-2 gene (phosphodiester or phosphorothioate)	
	GGT GCT CAC TGC GGC	(SEQ ID. No 3)
LR-3280	human c-myc gene (phosphorothioate)	
	AAC GTT GAG GGG CAT	(SEQ ID. No 4)
Inx-6298	human c-myc gene (phosphodiester)	
	AAC GTT GAG GGG CAT	(SEQ ID. No 4)
Inx-6295	human c-myc gene (phosphodiester or phosphorothioate)	
	T AAC GTT GAG GGG CAT	(SEQ ID. No 5)
LR-3001	human c-myb gene (phosphodiester or phosphorothioate)	
	TAT GCT GTG CCG GGG TCT TCG GGC	(SEQ ID. No 6)
c-myb	human c-myb gene (phosphodiester or phosphorothioate)	
	GTG CCG GGG TCT TCG GGC	(SEQ ID. No 7)

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IGF-1R	human IGF-1R (Insulin Growth Factor 1 – Receptor)	
	(phosphodiester or phosphorothioate)	
	GGA CCC TCC TCC GGA GCC	(SEQ ID. No 8)
LR-42	human IGF-1R (phosphodiester or phosphorothioate)	
5	TCC TCC GGA GCC AGA CTT	(SEQ ID. No 9)
EGFR	human EGFR (Epidermal Growth Factor Receptor) (phosphodiester or phosphorothioate)	
	CCG TGG TCA TGC TCC	(SEQ ID. No 10)
VEGF 10	human VEGF (Vascular Endothelial Growth Factor) gene (phosphodiester or phosphorothioate)	
	CAG CCT GGC TCA CCG CCT TGG	(SEQ ID. No 11)
PS-4189	murine PKC-alpha (Phosphokinase C – alpha) gene (phosphodiester or phosphorothioate)	
	CAG CCA TGG TTC CCC CCA AC	(SEQ ID. No 12)
15 PS-3521	human PKC-alpha (phosphodiester or phosphorothioate)	
	GTT CTC GCT GGT GAG TTT CA	(SEQ ID. No 13)
Bcl-2	human bcl-2 gene (phosphodiester or phosphorothioate)	
20	TCT CCC AgC gTg CgC CAT	(SEQ ID. No 14)
ATG-AS	human c-*raf*-1 protein kinase (phosphodiester or phosphorothioate)	
	GTG CTC CAT TGA TGC	(SEQ ID. No 15)
VEGF-R1 25	human VEGF-R –1 (Vascular Endothelial Growth Factor Receptor 1) ribozyme	
	GAG UUG CUG AUG AGG CCG AAA GGC CGA AAG UCU G	
		(SEQ ID. No 16)

Using these sequences, the invention provides a method for the treatment of a

diseases, including tumors, characterized by aberrant expression of a gene in a mammalian

30   subject. The method comprises the steps of preparing a lipid-encapsulated therapeutic

nucleic acid particle according to the methods as described herein, where the therapeutic

nucleic acid component hybridizes specifically with the aberrantly expressed gene; and

administering a therapeutically effective amount of the resulting particle to the mammalian

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subject. These sequences are, of course, only representative of the possible therapeutic oligonucleotide compounds that can be delivered using the invention. It is well known that, depending on the target gene, antisense that hybridizes to any part of the target gene, such as coding regions, introns, the 5' untranslated region (5'UTR), start of translation, or 3'UTR

5 may have therapeutic utility. Therefore, the sequences listed above are only exemplary of antisense. Furthermore, all the alternative chemistries that have been proposed (i.e. see Background) can be tested with the invention to determine efficacy along with all types of ribozymes. In short, the compounds listed above represent the broad class of therapeutic 5-50 mer oligonucleotides of various chemistries which are useful with this invention. Other

10 oligonucleotides which are useful include all those which have previously demonstrated efficacy in the free form.

While the invention is generally described and exemplified with regard to antisense oligonucleotides, other nucleic acids can be formulated and administered to a subject for the purpose of repairing or enhancing the expression of a cellular protein.

15 Accordingly, the nucleic acid can be an expression vector, cloning vector or the like which is often a plasmid designed to be able to replicate in a chosen host cell. Expression vectors may replicate autonomously, or they may replicate by being inserted into the genome of the host cell, by methods well known in the art. Vectors that replicate autonomously will have an origin of replication or autonomous replicating sequence (ARS) that is functional in the

20 chosen host cell(s). Often, it is desirable for a vector to be usable in more than one host cell, e.g., in *E. coli* for cloning and construction, and in a mammalian cell for expression.

Additionally, the nucleic acid can carry a label (*e.g.,* radioactive label, fluorescent label or colorimetric label) for the purpose of providing clinical diagnosis relating to the presence or absence of complementary nucleic acids. Accordingly, the nucleic acids,

25 or nucleotide polymers, can be polymers of nucleic acids including genomic DNA, cDNA, mRNA or oligonucleotides containing nucleic acid analogs, for example, the antisense derivatives described in a review by Stein, *et al., Science* **261**:1004-1011 (1993) and in U.S. Patent Nos. 5,264,423 and 5,276,019, the disclosures of which are incorporated herein by reference. Still further, the nucleic acids may encode transcriptional and translational

30 regulatory sequences including promoter sequences and enhancer sequences.

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The nucleic acids used in the present invention will also include those nucleic acids in which modifications have been made in one or more sugar moieties and/or in one or more of the pyrimidine or purine bases. Examples of sugar modifications include replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, azido

5   groups or functionalized as ethers or esters. Additionally, the entire sugar may be replaced with sterically and electronically similar structures, including aza-sugars and carbocyclic sugar analogs. Modifications in the purine or pyrimidine base moiety include, for example, alkylated purines and pyrimidines, acylated purines or pyrimidines, or other heterocyclic substitutes known to those of skill in the art. As with the modifications to the phosphodiester

10   linkages discussed above, any modifications to the sugar or the base moieties should also act to preserve at least a portion of the negative charge normally associated with the nucleic acid. In particular, modifications will preferably result in retention of at least 10% of the overall negative charge, more preferably over 50% of the negative charge and still more preferably over 80% of the negative charge associated with the nucleic acid.

15   The nucleic acids used in the present method can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries or prepared by synthetic methods. Synthetic nucleic acids can be prepared by a variety of solution or solid phase methods. Generally, solid phase synthesis is preferred. Detailed descriptions of the procedures for solid phase synthesis of nucleic acids by phosphite-triester, phosphotriester,

20   and H-phosphonate chemistries are widely available. See, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers, *et al.*, U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage, *et al.*, *Tetrahedron Lett.*, **22**:1859-1862 (1981); Matteucci, *et al.*, *J. Am. Chem. Soc.*, **103**:3185-3191 (1981); Caruthers, *et al.*, *Genetic Engineering*, **4**:1-17 (1982); Jones, chapter 2, Atkinson, *et al.*, chapter 3, and Sproat, *et al.*, chapter 4, in *Oligonucleotide Synthesis: A Practical*

25   *Approach*, Gait (ed.), IRL Press, Washington D.C. (1984); Froehler, *et al.*, *Tetrahedron Lett.*, **27**:469-472 (1986); Froehler, *et al.*, *Nucleic Acids Res.*, **14**:5399-5407 (1986); Sinha, *et al.* *Tetrahedron Lett.*, **24**:5843-5846 (1983); and Sinha, *et al.*, *Nucl. Acids Res.*, **12**:4539-4557 (1984) which are incorporated herein by reference.

As noted above, the solution of therapeutic agent (nucleic acids) comprises an

30   aqueous buffer. Preferred buffers (in the case of anionic therapeutic agents) are those which provide a pH of less than the pK_a of the first lipid component. Examples of suitable buffers

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include citrate, phosphate, acetate, and MES. A particularly preferred buffer is citrate buffer.

Preferred buffers will be in the range of 1-1000 mM of the anion, depending on the chemistry of the oligonucleotide being encapsulated, and optimization of buffer concentration may be significant to achieving high loading levels (See. Figs 15 and 20).   Alternatively,

5   pure water acidified to pH 5-6 with chloride, sulfate or the like may be useful. In this case, it may be suitable to add 5% glucose, or another non-ionic solute which will balance the osmotic potential across the particle membrane when the particles are dialyzed to remove ethanol, increase the pH, or mixed with a pharmaceutically acceptable carrier such as normal saline. The amount of therapeutic agent (nucleic acid) in buffer can vary, but will typically

10   be from about 0.01 mg/mL to about 200 mg/mL, more preferably from about 0.5 mg/mL to about 50 mg/mL.

The mixture of lipids and the buffered aqueous solution of therapeutic agent (nucleic acids) is combined to provide an intermediate mixture. The intermediate mixture is typically a mixture of lipid particles having encapsulated therapeutic agent (nucleic acids).

15   Additionally, the intermediate mixture may also contain some portion of therapeutic agent (nucleic acids) which are attached to the surface of the lipid particles (liposomes or lipid vesicles) due to the ionic attraction of the negatively-charged nucleic acids and positively-charged lipids on the lipid particle surface (the amino lipids or other lipid making up the protonatable first lipid component are positively charged in a buffer having a pH of less than

20   the pK_a of the protonatable group on the lipid). In one group of preferred embodiments, the mixture of lipids is an alcohol solution of lipids and the volumes of each of the solutions is adjusted so that upon combination, the resulting alcohol content is from about 20% by volume to about 45% by volume. The method of combining the mixtures can include any of a variety of processes, often depending upon the scale of formulation produced. For example,

25   when the total volume is about 10-20 mL or less, the solutions can be combined in a test tube and stirred together using a vortex mixer. Large-scale processes can be carried out in suitable production scale glassware.

Optionally, the lipid-encapsulated therapeutic agent (nucleic acid) complexes which are produced by combining the lipid mixture and the buffered aqueous solution of

30   therapeutic agents (nucleic acids) can be sized to achieve a desired size range and relatively narrow distribution of lipid particle sizes. Preferably, the compositions provided herein will

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be sized to a mean diameter of from about 70 to about 200 nm, more preferably about 90 to about 130 nm. Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive

5   size reduction down to small unilamellar vesicles (SUVs) less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size

10   distribution can be monitored by conventional laser-beam particle size determination. For the methods herein, extrusion is used to obtain a uniform vesicle size.

Extrusion of liposome compositions through a small-pore polycarbonate membrane or an asymmetric ceramic membrane results in a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times

15   until the desired liposome complex size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. In some instances, the lipid-nucleic acid compositions which are formed can be used without any sizing.

The present invention further comprises a step of neutralizing at least some of

20   the surface charges on the lipid portions of the lipid-nucleic acid compositions. By at least partially neutralizing the surface charges, unencapsulated antisense or other nucleic acid is freed from the lipid particle surface and can be removed from the composition using conventional techniques. Preferably, unencapsulated and surface adsorbed nucleic acids is removed from the resulting compositions through exchange of buffer solutions. For example,

25   replacement of a citrate buffer (pH about 4.0, used for forming the compositions) with a HEPES-buffered saline (HBS pH about 7.5) solution, results in the neutralization of liposome surface and antisense release from the surface. The released antisense can then be removed via chromatography using standard methods, and then switched into a buffer with a pH above the pKa of the lipid used.

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In other aspects, the present invention provides lipid-encapsulated nucleic acid compositions, preferably prepared by the methods recited above. Accordingly, preferred compositions are those having the lipid ratios and nucleic acid preferences noted above.

In still other aspects, the present invention contemplates reversed-charge
5   methods in which the lipid portion of the complex contains certain anionic lipids and the component which is encapsulated is a positively charged therapeutic agent. One example of a positively charged agent is a positively charged peptide or protein. In essentially an identical manner, liposome-encapsulated protein is formed at a pH above the pKa of the anionic lipid, then the surface is neutralized by exchanging the buffer with a buffer of lower pH (which
10   would also release surface-bound peptide or protein).

IV.   Pharmaceutical Preparations

The lipid-nucleic acid compositions prepared by the above methods can be administered either alone or in mixture with a physiologically-acceptable carrier (such as
15   physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice.

Pharmaceutical compositions comprising the lipid-nucleic acid compositions of the invention are prepared according to standard techniques and further comprise a pharmaceutically acceptable carrier. Generally, normal saline will be employed as the
20   pharmaceutically acceptable carrier. Other suitable carriers include, e.g., water, buffered water, 0.9% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* In compositions comprising saline or other salt containing carriers, the carrier is preferably added following lipid particle formation. Thus, after the lipid-nucleic acid compositions are formed, the compositions can be diluted into
25   pharmaceutically acceptable carriers such as normal saline. The resulting pharmaceutical preparations may be sterilized by conventional, well known sterilization techniques. The aqueous solutions can then be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary
30   substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium

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lactate, sodium chloride, potassium chloride, calcium chloride, *etc.* Additionally, the lipidic suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as α-tocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

5   The concentration of lipid-nucleic acid complexes in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.01%, usually at or at least about 0.05-5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with 10   treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, complexes composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration. In one group of embodiments, the nucleic acid will have an attached label and will be used for diagnosis (by indicating the presence of complementary nucleic acid). In 15   this instance, the amount of complexes administered will depend upon the particular label used, the disease state being diagnosed and the judgement of the clinician but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight.

        As noted above, the lipid-therapeutic agent (nucleic acid) compositions of the 20   invention include polyethylene glycol (PEG)-modified phospholipids, PEG-ceramide, or ganglioside G_{M1}-modified lipids or other lipids effective to prevent or limit aggregation. Addition of such components does not merely prevent complex aggregation, however, it may also provides a means for increasing circulation lifetime and increasing the delivery of the lipid-nucleic acid composition to the target tissues.

25   The present invention also provides lipid-nucleic acid compositions in kit form. The kit will typically be comprised of a container which is compartmentalized for holding the various elements of the kit. The kit will contain the compositions of the present inventions, preferably in dehydrated or concentrated form, with instructions for their rehydration or dilution and administration. In still other embodiments, the lipid-30   encapsulated-therapeutic agent (nucleic acid) particles will have a targeting moiety attached to the surface of the lipid particle. Methods of attaching targeting moieties (*e.g.*, antibodies,

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proteins, small molecule mimetics, vitamins, oligosaccharides and hyaluronic acid) to lipids (such as those used in the present compositions) are known to those of skill in the art.

Dosage for the lipid-nucleic acid compositions will depend on the ratio of nucleic acid to lipid and the administrating physician's opinion based on age, weight, and condition of the patient.

V.   Methods of Introducing Lipid-Encapsulated Therapeutic Agents Into Cells

The lipid-therapeutic agent compositions of the invention can be used for introduction of those therapeutic agents into cells. In the case of nucleic acid-containing compositions, the composition of the invention are useful for the introduction of nucleic acids, preferably plasmids, antisense and ribozymes into cells. Accordingly, the present invention also provides methods for introducing a therapeutic agent such as a nucleic acid into a cell. The methods are carried out *in vitro* or *in vivo* by first forming the compositions as described above, then contacting the compositions with the target cells for a period of time sufficient for transfection to occur.

The compositions of the present invention can be adsorbed to almost any cell type. Once adsorbed, the complexes can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the nucleic acid portion of the complex can take place via any one of these pathways. In particular, when fusion takes place, the liposome membrane is integrated into the cell membrane and the contents of the liposome combine with the intracellular fluid. Contact between the cells and the lipid-nucleic acid compositions, when carried out *in vitro*, will take place in a biologically compatible medium. The concentration of compositions can vary widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. Treatment of the cells with the lipid-nucleic acid compositions will generally be carried out at physiological temperatures (about 37°C) for periods of time of from about 1 to 6 hours, preferably of from about 2 to 4 hours. For *in vitro* applications, the delivery of nucleic acids can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells will be animal cells, more preferably mammalian cells, and most preferably human cells.

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     In one group of preferred embodiments, a lipid-nucleic acid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/mL, more preferably about 2×10^4 cells/mL. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 µg/mL, more preferably about 0.1 µg/mL.

     Typical applications include using well known transfection procedures to provide intracellular delivery of DNA or mRNA sequences which code for therapeutically useful polypeptides. In this manner, therapy is provided for genetic diseases by supplying deficient or absent gene products (*i.e.,* for Duchenne's dystrophy, see Kunkel, *et al., Brit. Med. Bull.* **45(3):**630-643 (1989), and for cystic fibrosis, see Goodfellow, *Nature* **341:**102-103 (1989)). Other uses for the compositions of the present invention include introduction of antisense oligonucleotides in cells (see, Bennett, *et al., Mol. Pharm.* **41:**1023-1033 (1992)).

     Alternatively, the compositions of the present invention can also be used for the transfection of cells *in vivo*, using methods which are known to those of skill in the art. In particular, Zhu, *et al., Science* **261:**209-211 (1993), incorporated herein by reference, describes the intravenous delivery of cytomegalovirus (CMV)-chloramphenicol acetyltransferase (CAT) expression plasmid using DOTMA-DOPE complexes. Hyde, *et al., Nature* **362:**250-256 (1993), incorporated herein by reference, describes the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to epithelia of the airway and to alveoli in the lung of mice, using liposomes. Brigham, *et al., Am. J. Med. Sci.* **298:**278-281 (1989), incorporated herein by reference, describes the *in vivo* transfection of lungs of mice with a functioning prokaryotic gene encoding the intracellular enzyme, chloramphenicol acetyltransferase (CAT). Thus, the compositions of the invention can be used in the treatment of infectious diseases.

     For *in vivo* administration, the pharmaceutical compositions are preferably administered parenterally, *i.e.,* intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. More preferably, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection. For example, see Stadler, *et al.,* U.S. Patent No. 5,286,634, which is incorporated herein by reference. Intracellular nucleic acid delivery has also been discussed in Straubringer, *et al.,* METHODS IN ENZYMOLOGY, Academic Press, New York. **101:**512-527 (1983); Mannino, *et al.,*

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Biotechniques **6**:682-690 (1988); Nicolau, *et al., Crit. Rev. Ther. Drug Carrier Syst.* **6**:239-271 (1989), and Behr, *Acc. Chem. Res.* **26**:274-278 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, Rahman *et al.,* U.S. Patent No. 3,993,754; Sears, U.S. Patent No. 4,145,410; Papahadjopoulos *et al.,* U.S. Patent

5 No. 4,235,871; Schneider, U.S. Patent No. 4,224,179; Lenk *et al.,* U.S. Patent No. 4,522,803; and Fountain *et al.,* U.S. Patent No. 4,588,578.

In other methods, the pharmaceutical preparations may be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or "closed" procedures. By "topical", it is meant the direct

10 application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those procedures which include incising the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to

15 access abdominal viscera, or other direct surgical approach to the target tissue. "Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage. Likewise, the pharmaceutical preparations may be administered to the meninges or spinal

20 cord by infusion during a lumbar puncture followed by appropriate positioning of the patient as commonly practiced for spinal anesthesia or metrazamide imaging of the spinal cord. Alternatively, the preparations may be administered through endoscopic devices.

The lipid-nucleic acid compositions can also be administered in an aerosol inhaled into the lungs (see, Brigham, *et al., Am. J. Sci.* **298(4)**:278-281 (1989)) or by direct

25 injection at the site of disease (Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71 (1994)).

The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as humans, non-human primates, dogs, cats, cattle, horses, sheep, and the like.

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VI.   Examples

Materials and Methods:

Lipids

Distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), and
5   palmitoyloleoylphosphatidylcholine (POPC) were purchased from Northern Lipids
(Vancouver, Canada). 1,2-dioleoyloxy-3-dimethylammoniumpropane (DODAP or AL-1)
was synthesized by Dr. Steven Ansell (Inex Pharmaceuticals) or, alternatively, was purchased
from Avanti Polar Lipids. Cholesterol was purchased from Sigma Chemical Company (St.
Louis, Missouri, USA). PEG-ceramides were synthesized by Dr. Zhao Wang at Inex
10   Pharmaceuticals Corp. using procedures described in PCT WO 96/40964, incorporated herein
by reference. [^{3}H] or [^{14}C]-CHE was purchased from NEN (Boston, Massachusetts, USA).
All lipids were > 99% pure.

Buffers and Solvents

15   Ethanol (95%), methanol, chloroform, citric acid, HEPES and NaCl were all
purchased from commercial suppliers.

Synthesis and Purification of Phosphorothioate Antisense

PS 3082, a 20mer phosphorothioate antisense oligodeoxynucleotide, was
20   synthesized, purified and donated by ISIS Pharmaceuticals (Carlsbad, California, USA). The
sequence for this oligo is: TGCATCCCCCAGGCCACCAT. (Seq ID No 1) The details of
the synthesis and purification can be found elsewhere (see, Stepkowski, *et al., J. Immunol.*
153:5336-5346 (1994)).

Preparation of Liposomal Antisense

25   Lipid stock solutions were prepared in 95% ethanol at 20 mg/mL
(PEG-Ceramides were prepared at 50 mg/mL). DSPC, CHOL, DODAP, PEG-CerC14
(25:45:20:10, molar ratio), 13 µmol total lipid, were added to a 13 x 100 mm test tube
containing trace amounts of [^{14}C]-cholesterylhexadecylether. The final volume of the lipid
30   mixture was 0.4 mL. In some experiments, SM or POPC was substituted for DSPC. A
20mer antisense oligodeoxynucleotide, PS 3082 (2 mg), and trace amounts of [^{3}H]-PS 3082

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were dissolved in 0.6 mL of 300 mM citric acid, pH 3.8 in a separate 13 x 100 mm test tube. The antisense solution was warmed to 65 °C and the lipids (in ethanol) were slowly added, mixing constantly. The resulting volume of the mixture was 1.0 mL and contained 13 μmol total lipid, 2 mg of antisense oligodeoxynucleotide, and 38% ethanol, vol/vol. The

5   antisense-lipid mixture was subjected to 5 cycles of freezing (liquid nitrogen) and thawing (65 °C), and subsequently was passed 10X through three stacked 100 nm filters (Poretics) using a pressurized extruder apparatus with a thermobarrel attachment (Lipex Biomembranes). The temperature and pressure during extrusion were 65 °C and 300-400 psi (nitrogen), respectively. The extruded preparation was diluted with 1.0 mL of 300 mM citric

10   acid, pH 3.8, reducing the ethanol content to 20%. The preparation was immediately applied to a gel filtration column. Alternatively, the extruded sample was dialyzed (12 000-14 000 MW cutoff; SpectraPor) against several liters of 300 mM citrate buffer, pH 3.8 for 3-4 hours to remove the excess ethanol. The sample was subsequently dialyzed against HBS, pH 7.5, for 12-18 hours to neutralize the DODAP and release any antisense that was associated with

15   the surface of the vesicles. The free antisense was removed from the encapsulated liposomal antisense by gel exclusion chromatography as described below.

Gel Filtration Chromatography

A 20 x 2.5 cm glass column containing Biogel A15m, 100-200 mesh, was

20   equilibrated in HEPES-buffered saline (HBS; 20 mM HEPES, 145 mM NaCl, pH 7.5). The 2.0 mL liposomal antisense preparation was applied to the column and allowed to drain into the gel bed under gravity. The column was eluted with HBS at a flow rate of 50 mL/hr. Column fractions (1.0 mL) were collected and analyzed for radioactivity using standard liquid scintillation counting techniques. The fractions were pooled based on the levels of

25   [14C]-CHE present in the fraction. The size distribution of the pooled liposomal antisense was determined using a NICOMP Model 370 Sub-micron particle sizer and was typically 110 ± 30 nm.

Ion Exchange Chromatography

30   As an alternative to gel filtration chromatography, samples were sometimes dialyzed first in 300 mM citrate, pH 3.80, for 2-3 hours to remove residual ethanol, followed

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by at least a 12 hour dialysis in HBS, to exchange the external citrate for HBS and remove residual ethanol. The sample was applied to a 1.5 x 8 cm DEAE-Sepharose® column equilibrated in HBS. Free oligonucleotide binds to the DEAE with very high affinity. The peak containing the lipid was pooled, concentrated, and analyzed for antisense content, as
5   described below.

Assessment of Antisense Encapsulation

Antisense encapsulation was typically assessed by dual label ([^{3}H]-antisense and [^{14}C]-lipid) liquid scintillation counting after gel filtration chromatography to separate the
10   free and encapsulated antisense. Antisense encapsulation was evaluated by summing the total [^{3}H]-antisense radioactivity associated with the lipid peak and dividing by the total [^{3}H]-antisense radioactivity. Alternatively, the [^{3}H]/[^{14}C] ratio was determined before and after (*i.e.*, in the pooled lipid peak) gel filtration chromatography. Antisense encapsulation was also assessed by measuring the absorbance of the sample at 260 nm, preceded by a Bligh
15   and Dyer extraction of the antisense from the lipid, as described below.

Extraction of the Antisense

The antisense was extracted from the lipid according to the procedure outlined by Bligh and Dyer (Bligh, *et al.*, *Can. J. Biochem. Physiol.* **37**:911-917 (1959)). Briefly, up
20   to 250 µL of aqueous sample was added to a 13 x 100 mm glass test tube, followed by the addition of 750 µL of chloroform:methanol (1:2.1, vol/vol), 250 µL of chloroform, and 250 µL of distilled water. The sample was mixed after each addition. The sample was centrifuged for 10 min. at 3000 rpm, resulting in a clear two-phase separation. The aqueous phase (top) was removed into a new 13 x 100 mm test tube. An aliquot (500 µL) of this
25   phase was diluted with 500 µL of distilled water, mixed, and the absorbance at 260 nm was assessed using a spectrophotometer. In some instances, the organic phase (bottom) was washed with 250 µL of methanol, centrifuged for 10 min. at 3000 rpm, and the upper phase removed and discarded. This was repeated 3 times. The washed organic phase was assessed for phospholipid content according to the method of Fiske and Subbarrow (Fiske, *et al.*, *J.*
30   *Biol. Chem.* **66**:375-400 (1925)).

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OLIGREEN Assay

A fluorescent dye binding assay for quantifying single stranded oligonucleotide in aqueous solutions was established using a Biolumin™ 960 fluorescent plate reader (Molecular Dynamics, Sunnyvale, California, USA). Briefly, aliquots of

5   encapsulated oligonucleotide were diluted in HEPES buffered saline (HBS; 20mM HEPES, 145mM NaCl, pH 7.5) . A 10 µL aliquot of the diluted sample was added to 100 µL of a 1:200 dilution of Oligreen™ reagent, both with and without 0.1% of Triton X-100 detergent. An oligo standard curve was prepared with and without 0.1% Triton X-100 for quantification of encapsulated oligo. Fluorescence of the OLIGREEN™-antisense complex was measured

10   using excitation and emission wavelengths of 485nm and 520nm, respectively. Surface associated antisense was determined by comparing the fluorescence measurements in the absence and presence of detergent.

Ear Inflammation Model and Efficacy Studies

15   #### Sensitization and Elicitation of Contact Sensitivity

Mice were sensitized by applying 25 µL of 0.5% 2,4-dinitro-1-fluorobenzene (DNFB) in acetone:olive oil (4:1) to the shaved abdominal wall for two consecutive days. Four days after the second application, mice were challenged on the dorsal surface of the left ear with 10 µL of 0.2% DNFB in acetone:olive oil (4:1). Mice received no treatment on the

20   contralateral (right) ear. In some cases, control mice received 10 µL of vehicle on the dorsal surface of the left ear.

Evaluation of Ear Swelling

Ear thickness was measured immediately prior to ear challenge, and at various time intervals after DNFB challenge, using an engineer's micrometer (Mitutoyo, Tokyo,

25   Japan). Increases in ear thickness measurements were determined by subtracting the pre-challenge from post-challenge measurements.

The progression of ear inflammation over a 3 day period for ICR (outbred) mice is indicated in Figures 12 and 13. Erythema was evident almost immediately after ear challenge and gradually declined in intensity over the remainder of the study. ICR mice

30   exhibited peak ear thickness at 24 hours after the induction of ear inflammation. Maximal ear thickness measurements were found to be 170×10^{-4} inches, corresponding to a 70% increase

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in ear thickness. Although ear swelling gradually declines at 48 and 72 hours after

inflammation initiation, ear measurements still have not returned to baseline thickness levels

$(90-100 \times 10^{-4}$ inches).

The mouse *in vivo* experimental systems in this specification were selected in

5   part because of their high degree of correlation to human disease conditions. The mouse ear

inflammation model , which can be treated using methods and compositions of the invention,

is well known to be an excellent model for human allergic contact dermatitis and other

disease conditions. The control therapeutic used in this model is a corticosteroid which

demonstrates efficacy both in the mouse model and in related human disease conditions.

10   The mouse B16 tumor model, a fast growing melanoma, which can be treated

using methods and compositions of the invention, is a standard, widely used experimental

system. This tumor model can be successfully treated using vinca alkaloids, such as

vincristine or vinblastine, which are known to be efficacious against human tumors as well.

Treatments which demonstrate utility in the mouse models of this invention

15   are excellent candidates for testing against human disease conditions, at similar dosages and

administration modalities.

EXAMPLE 1

This example illustrates the effects of ethanol on the encapsulation of

20   antisense.

A 20mer of $[^3H]$-phosphorothioate antisense oligodeoxynucleotide (in 300

mM citrate buffer, pH 3.80) was mixed with an ethanol solution of lipid

(DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio) at final concentrations of 2

mg/mL and 9.9 mg/mL, respectively. The final ethanol concentration in the preparations was

25   varied between 0 and 60%, vol/vol. The samples were extruded ten times through three 100

nm filters as described in "Materials and Methods". The samples were dialyzed for 2-3 hours

in 300 mM citrate buffer, pH 3.80, to remove a majority of the excess ethanol. The samples

were switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12

hours to replace the external citrate buffer with HBS. This renders the majority of DODAP in

30   the outer bilayer neutral, and will release any surface bound antisense. Non-encapsulated

antisense was then removed from the liposomal antisense by DEAE-sepharose

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chromatography as described in "Material and Methods". Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [³H]-antisense and [¹⁴C]-lipid or by determining the total pre-column and post-column [³H]-antisense and [¹⁴C]-lipid radioactivity.

5   In another experiment, the formulations were prepared as described. After extrusion, the filters were analyzed for [³H]-antisense and [¹⁴C]-lipid radioactivity by standard scintillation counting techniques. Results were expressed as a percent of the total initial radioactivity.

           Figure 3 demonstrates the effects of ethanol on the encapsulation of antisense

10   at pH 3.8. The encapsulation efficiency of phosphorothioate antisense increases in a near linear manner up to a final ethanol concentration of 50%, vol/vol. At an ethanol content greater than 50%, a large amount of aggregation/precipitation is observed. The effect of ethanol on vesicle formation can be further observed by monitoring both lipid and antisense loss on the filters during extrusion (Figure 4). At low ethanol contents, extrusion is slow and

15   the proportion of lipid and antisense loss is the same, suggesting that the losses are due to the formation of large complexes which get trapped on the filter. At ethanol contents of 30 and 40%, extrusion is very quick and losses of both lipid and antisense are minimal. As the ethanol content is increased above 40%, the loss of antisense becomes disproportionally high relative to the lipid. This can be attributed to the insolubility of DNA in high concentrations

20   of alcohol. Furthermore, in the presence of ethanol, PEG is required to prevent aggregation and fusion of the vesicles (results not shown).

EXAMPLE 2

           This example illustrates the effects of DODAP on the encapsulation of anti-

25   sense, and further illustrates the effect of initial antisense concentration on the compositions.

           Having demonstrated that ethanol can greatly facilitate the preparation of lipid vesicles containing entrapped antisense, the next step was to examine the influence of DODAP (AL-1) content on the encapsulation of antisense (Figure 5). Accordingly, a 0.6 mL aliquot of a [³H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with 0.4 mL of a 95% ethanol solution of lipid (DSPC:CHOL:DODAP:

30   PEG-CerC14; 100-(55+X):45:X:10, molar ratio) at final concentrations of 2 mg/mL and 9.9

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mg/mL, respectively. The molar ratio of DODAP was varied between 0 and 30%. The molar ratio of DSPC was adjusted to compensate for the changes in DODAP content. The samples were extruded ten times through three 100 nm filters as described in "Materials and Methods", and were dialyzed for 2-3 hours in 300 mM citrate buffer, pH 3.80, to remove a

5 majority of the excess ethanol. The samples were switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods". Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and

10 [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity. As seen in Figure 5, antisense encapsulation increased significantly between 5-20% DODAP. At DODAP contents greater than 20-25%, extrusion of the vesicles became more difficult suggesting the formation of complexes. At DODAP concentration of 40 and 50%, extrusion of the lipid / antisense mixture took hours compared to minutes for a

15 lipid composition containing 20% DODAP. To verify that the antisense was indeed associated with the lipid and that the observed encapsulation was not due to exchange of the [^{3}H]-label from the antisense onto the lipid, the antisense was extracted from the lipid using a Bligh and Dyer extraction. Using this technique, the antisense, which is soluble in the aqueous phase, was separated from the lipid (soluble in the organic phase) and quantified by

20 measuring the absorbance at 260 nm (Figure 6). While this method can underestimate the antisense concentration, the technique substantiated that the observed association of antisense with the lipid was not an artifact.

In yet another experiment, varying concentrations of a 20mer of [^{3}H]-phos-phorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) were mixed

25 with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio), 9.9 mg/mL (final concentration). The samples were extruded and dialyzed twice as described above. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods". Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and

30 [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and

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[^{14}C]-lipid radioactivity. EPC:CH liposomes containing encapsulated antisense are included for comparison.

Optimization of the drug:lipid ratio was accomplished by increasing the initial antisense concentration that was mixed with 9.8 mg total lipid (DSPC:CHOL:DODAP: PEG-CerC14; 25:45:20:10) (Figure 8). Drug:lipid ratios of up to 0.25, w/w, were obtained using 10 mg/mL of antisense in the preparation. However, the increased drug:lipid ratio was accompanied by a decrease in encapsulation efficiency, therefore a compromise must be made between optimizing the drug:lipid ratio and encapsulation efficiency. In comparison, antisense encapsulated by hydration of a dry lipid film (i.e. EPC:CHOL) in the absence of cationic lipid typically yields low encapsulation efficiencies (< 12-15%) and drug:lipid ratios (< 0.1, w/w). Consequently, significant quantities of antisense are wasted during the encapsulation procedure.

EXAMPLE 3

This example illustrates the properties of the liposomal antisense formulations provided in the Materials and Methods above.

The size distribution of a liposomal preparation of antisense was determined by quasi-elastic light scattering (QELS) immediately after removal of the free antisense (A), and after storage of the preparation for 2 months at 4°C (B), using a Nicomp Model 370 sub-micron particle sizer. A 0.6 mL aliquot of a [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with 0.4 mL of a 95% ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The sample was extruded ten times through three 100 nm filters as described in "Materials and Methods", and dialyzed for 2-3 hours in 300 mM citrate buffer, pH 3.80, to remove a majority of the excess ethanol. The sample was switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods".

The size distribution and storage stability of antisense preparations described herein is demonstrated in Figure 7. The size distribution of a standard

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DSPC:CHOL:DODAP:PEG-CerC14 (25:45:20:10) preparation containing a 2 mg/mL initial antisense concentration was analyzed immediately after column chromatography to remove any free antisense. A very homogenous distribution is observed after preparation (119 ± 32 nm). This size distribution remained stable for at least 2 months after storage at 4°C (119 ± 32 nm).

EXAMPLE 4

This example illustrates the clearance pharmacokinetics, biodistribution and biological activity of an encapsulated murine ICAM-1 phosphorothioate antisense oligodeoxynucleotide.

4.1   *Plasma clearance*

Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP:PEG-CerC14 (25:45:20:10), where X represents either distearoyl-phosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 µL) intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

The plasma clearance of three formulations, DSPC:CHOL:DODAP:PEG-CerC14, SM:CHOL:DODAP:PEG-CerC14, and POPC:CHOL:DODAP:PEG-CerC14, of encapsulated antisense were examined in inflamed ICR mice (Figure 9). The circulation time was longest for the DSPC version of the formulation.

4.2   *Organ accumulation*

Liposomal antisense compositions were prepared and administered to mice as outlined in the preceding section. Mice were terminated by cervical dislocation and the

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organs were recovered and processed as described in "Materials and Methods". Lipid and antisense recoveries were determined by standard scintillation counting techniques.

Organ accumulation of the various formulations was typical of previously described liposome clearance patterns, with the RES organs, principally the liver and spleen,

5   being responsible for the majority of clearance (Figure 10). One interesting observation is that the liver and spleen clearance account for only 40-45% of the total clearance of the "DSPC" formulation, suggesting that a significant population of vesicles is accumulating in another organ system or is being excreted.

10   *4.3   Stability.*

Liposomal antisense compositions were prepared and administered to mice as outlined in the preceding section. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques. Release rates were determined by measuring the [3H]/[14C] ratio over time.

15   The stability of the formulations was also assessed by measuring the ratio of antisense and lipid recovery in the blood at various times (Figure 11). A ratio of 1.0 suggests that the antisense and the lipid are staying together in the circulation. The "DSPC" formulation showed little deviation from a ratio of 1.0 over 24 h, suggesting that it is very stable in the circulation. The "POPC" formulation dropped to a ratio of 0.6 after 2 h, while

20   the ratio for the "SM" formulation decreased more slowly, reaching 0.6 after 12 h in the circulation. These results indicate that it may be possible to deliberately alter the antisense release rates by modifying the lipid composition.

4.4   PEG-Acyl Influence on circulation half-life of single dose of thioate antisense

25   Encapsulated lipid-encapsulated antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of DSPC:CHOL:DODAP:PEG-CerC14 or C20 (25:45:20:10). The formulation contained a lipid label ($[^{14}C]$-cholesterylhexadecylether) and $[^3H]$-antisense and were injected (200 μL)

30   intravenously via the lateral tail vein of female (20-25 g) ICR mice at a lipid dose of 120

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mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense
recoveries were determined by standard scintillation counting techniques.

The influence of PEG-acyl chain length on clearance rates of a
DSPC:CHOL:DODAP:PEG-Cer formulation was investigated using PEG-CerC14 and

5   PEG-CerC20 (**Figure 12**). The inclusion of PEG-CerC20 in the formulation resulted in
enhanced circulation times over the PEG-CerC14. This corresponds to in vitro data
suggesting that the C14 version of the PEG is exchanged much more rapidly out of the
vesicle than the C20 version.

10   *4.5   In vivo efficacy of single dose of lipid encapsulated ICAM-1 (phosphorothioate)
antisense*

The efficacy of PS- 3082 encapsulated in various lipid formulations containing
DODAP was tested in an ear inflammation model using ICR mice.

Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v.

15   dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped PS- 3082 (identified as
AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped PS- 3082 (identified as AS
4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped PS- 3082 (identified as AS
4200). Ear swelling was measured at 24 hours after initiating inflammation using an
engineer's micrometer.

20   Ear swelling measurements were made 24 hours after initiating inflammation
in mice treated i.v. at the time of ear challenge with either HBS (control), PS- 3082
encapsulated in EPC:CHOL vesicles (30 mg/kg dose of oligo), PS- 3082 encapsulated in
POPC:CHOL:DODAP:PEG-CerC14 vesicles (30 mg/kg dose of oligo), or PS- 3082
encapsulated in DSPC:CHOL:DODAP:PEG-CerC14 vesicles (30 mg/kg dose of oligo)

25   (**Figure 13**). The "DSPC" formulation resulted in the greatest efficacy, exhibiting only 10%
increase in ear swelling over pre-challenge values. A similar trend was observed for cellular
infiltration into the "challenged" ear versus the non-treated ear (**Figure 14**).

In another evaluation, mice received 10 μCi of [^{3}H]-methylthymidine, i.p., 24
hours before initiating inflammation. Inflamed mice were treated at the time of ear challenge

30   with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped
PS- 3082 (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped PS-

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3082 (identified as AS 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped PS-3082 (identified as AS 4200). Cell infiltration was monitored by measuring the radioactivity in the "challenged ear" versus the non-treated ear. Results are expressed as the ratio of radioactivity in the left (challenged ear) versus right ear.

5

4.6   In vivo efficacy of single dose of lipid encapsulated ICAM-1 (phosphodiester) antisense

This experiment demonstrates the *in vivo* efficacy of a phosphodiester antisense oligodeoxynucleotide encapsulated in lipid particles according to the invention. In specific,
10   the phosphodiester was targeted to the ICAM-1 gene in an ear inflammation model.

Group	Test Sample/Drug	Dose	Time Point
1	control inflammation - HBS	200 μl	24 hr
2	corticosteroid	200 μl	24 hr
3	empty vesicles	200 μl	24 hr
4	PS-3082	200 μl	24 hr
5	PO-3082	200 μl	24 hr

Antisense Sample Preparation: Antisense was encapsulated using the standard methods of Examples 5-9, using the phosphodiester modification. The phosphodiester formulation
20   used 10-50 mM citrate (preferably 20 mM citrate), pH 4.0 instead of 300 mM citrate, pH 4.0 preferred for phosphorothioates. Empty vesicles consisted of lipid components only. Corticosteroid (either Halobetasol propionate 0.05% by weight (Westwood Squibb, Montreal) or Dexamethasone (50 ug dissolved in 4:1 acetone:olive oil)) was applied topically in a thin film to cover the surface of the ear 15 minutes after ear challenge.

25   **Inflammation and Dosing:** Mouse ear inflammation was induced using DNFB as described above in Materials and Methods. Female ICR mice (6-8 weeks old) received intravenous tail vein injections of antisense (200 μl). Antisense doses for the phosphorothioate and phosphodiester antisense were adjusted to be 20-30 mg/kg. 6 mice were tested with each formulation. Administration occurred 15 min. after the application of 0.2% DNFB to the
30   mouse ear. Ear measurements were made on anaesthetized mice 24 hours after treatment (unless shown otherwise) and prior to termination. Mice are terminated by cervical dislocation and the ears are removed around the pinna. Ears are then weighted, digested

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(Solvable) and analyzed for radioactivity by liquid scintillation counting. Ears were analyzed for 1) Ear edema - based on the increase in ear thickness due to ear swelling. Calculated by subtracting pre-ear thickness values from post-ear thickness values **Figure 21**. 2) Cell infiltration - based on radioactivity accumulated in the inflamed (right) ear vs. the control

5   (left) ear **Figure 22**; and 3) Ear weights – left ear versus right ear (measurement of edema) **Figure 23**.

Results: The controls consisting of buffer alone (HBS) or Empty Vesicles alone demonstrated no efficacity. Topical corticosteroid demonstrates its known excellent efficacity by reducing inflammation to below pre-challenge levels. Both the

10   phosphorothioate and phosphodiester antisense show excellent efficacy through a systemic delivery administration, reducing the degree of inflammation by around 70% and 85%, respectively. Thus, it is possible to administer the compositions of the invention at a site where the disease site is distal to the site of the injection.

15   4.7   *In vivo efficacy of US3 antisense (Tumor Window Model)*

              In this example, the anti-tumor activity of lipid encapsulated US3, an antisense oligonucleotide directed at the erb-B-2 gene, has been demonstrated in an *in vivo* human breast tumor model.

              The human breast carcinoma line MDA-MB-453 was implanted in a mouse

20   tumor window model according to the method of Wu, N.Z., Da, D., Rudoll, T.L., Needham, D., Whorton, R. & Dewhirst, M.W. 1993. Increased microvascular permeability contributes to preferential accumulation of Stealth liposomes in tumor tissue. Cancer Research 53: 3765-3770; and Dewhirst, M.W., Tso, C.Y., Oliver, R., Gustafson, C.S., Secomb, T.W. & Gross, J.F. 1989. Morpholigic and hemodynamic comparison of tumor and healing normal tissue

25   microvasculature. Int. J. Radiat. Oncol. Biol. Phys. 17: 91-99. See also Dewhirst, MW., and Needham, D. 1995. Extravasation of Stealth Liposomes into Tumors: Direct Measurement of Accumulation and Vascular Permeability using a Skin Flap Window Chamber. In Stealth Liposomes (Eds. Lasic, D. and Martin, F.) CRC Press.

              The lipid-antisense formulation consists of disteroylphosphatidylcholine

30   (DSPC, 25 mol%), cholesterol (Chol, 45 mol%), dioleoylphosphatidyldiaminopropane, (DODAP, or AL1, 20 mol%) and PEG-ceramide (C14 chain length, 10 mol%). For some

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experiments detailed below, proportions and constituents were altered, but the method of preparation remained the same. Lipids were dissolved in ethanol at 20 mg/ml (PEG-ceramide at 50 mg/ml). Routinely, 1 to 2 μCi ^{14}C-cholesterylhexadecylether was added as a lipid radiolabel. Lipids were mixed in the correct proportions in ethanol to a final concentration of

5   10 mg in 400 μl. The lipid mixture was then added dropwise to phosphorothioated antisense (US3: anti-human erb-B-2 GGT GCT CAC TGC GGC (SEQ ID. No 3) dissolved in 300 mM citrate buffer pH 4.0 (600 μl to make a final volume of 1 ml). The antisense was used at a variety of concentrations, but the optimum concentration for maximum encapsulation efficiency and drug:lipid ratio was determined to be 0.5 mg/ml final. During the addition, the

10   solution becomes opaque. The DODAP is positively charged at pH 4.0 (pKa = 6.53) and so attracts the negatively charged DNA molecules. The mixture was subjected to five cycles of freezing in liquid N$_2$ and thawing at 65 °C followed by extrusion through 100 nm filters ten times at 65 °C.

After extrusion, two methods can be used for removal of the external

15   antisense. Firstly, the liposomes are diluted 2:1 with citrate (to reduce ethanol content to 20%) then applied to a Bio-Gel A18M 100-200 mesh column equilibrated with HBS. The column profiles shown in this report were generated in this manner. Alternatively, the liposomes are dialysed 2h against citrate to remove ethanol, the overnight against HBS to increase the external pH. The resulting mixture is then applied to a DEAE cation exchange

20   column to remove external oligo. This method was the routine method used for sample preparation for *in vivo* studies. Antisense concentrations were routinely determined by A260 measurements. Lipid concentrations were determined by scintillation counting after spiking initial mixture with a known concentration of ^{3}H or ^{14}C cholesterylhexadecyl ether, or by HPLC. Encapsulation efficiency was determined by division of the final drug to lipid ratio

25   by the initial drug to lipid ratio.

In vivo efficacy evaluation: When the tumor in the window has reached a diameter of 2-3 mm, treatment with free or TCS-encapsulated US3 oligonucleotide is initiated. Treatment consists of a 200 ul intravenous administration (tail vein) of either free US3 or TCS-encapsulated US3 on a 3 administrations/week schedule and an antisense dose

30   of 10 mg/kg/administration. Tumor size is monitored 3 times per week by microscopy.

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Results: The TCS-encapsulated US3 oligonucleotide was very effective at preventing the growth, or causing extensive size reduction, of the MDA-MB-453 human breast carcinoma in the window model. In contrast, unencapsulated oligonucleotide was ineffective at inhibiting tumor growth.

5

4.8   *In vivo clearance of various formulations using alternative amino lipids: DODAP or DODMA*

Antisense particle formulations were prepared according to Example 2, with the following modifications: In assay#1 and #2, 25% AL-1 (hydrochloride salt of DODAP) and 25% free base DODAP were employed, respectively, with a concomitant reduction in the amount of DSPC. Assay#3, 4 and 5 employed 30%, 25% and 20% DODMA (free base (prepared at Inex Pharmaceuticals Corp., Burnaby BC)), respectively, again with a concomitant reduction of DSPC.

Both the encapsulation efficiency and *in vivo* clearance of the formulations were studied. There was no significant difference between the encapsulation or clearance of the free base or HCl salt of DODAP. Decreasing DODMA concentration (30, 25, 20 %) severely decreased the encapsulation efficiency of PS-2302 (91%, 43%, 35%) and likewise the Drug/Lipid ratio of the resulting formulation.

In the clearance study outlined in **Figure 16**, DODMA formulations demonstrated slightly higher rates of clearance than 25 % DODAP or AL-1, although all formulations appear to be retained in the circulation to a degree which is suitable for human therapeutics.

4.9   *PEG-acyl influence on clearance rate of repeat doses of encapsulated EGF-R phosphorothioate antisense*

Lipid -encapsulated antisense was prepared using the ethanol-citrate procedure as described above, with changes to molar ratios of components as indicated. Initial lipid and antisense concentrations were about 9.9 and 2 mg/mL, respectively. DODAP containing formulations had drug:lipid ratios of 0.15 (+/-) 0.05. Passive encapsulation systems had drug:lipid ratios of 0.03. Nine different liposomal formulations were prepared, using standard techniques, in the following molar ratios:

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Formu-lation	DSPC (mol%)	Chol (mol%)	DODAP (mol%)	Steric Barrier Derivatized Lipid (name: mol%)	Antisense (EGF-R 2mg/ml)
1	55	45	Nil	Nil	Empty
2	50	45	Nil	ATTA8-DSPE : 5	Empty
3	50	45	Nil	ATTA8-DSPE : 5	AS
4	20	45	30	ATTA8-DSPE : 5	AS
5	20	45	30	PEG-DSPE : 5	AS
6	25	45	25	PEG-CerC14 : 5	Empty
7	25	45	25	PEG-CerC14 : 5	AS
8	25	45	25	PEG-CerC20 : 5	Empty
9	25	45	25	PEG-CerC20 : 5	AS

Antisense ("AS") used was fully phosphorothioated EGFR (anti-human Epidermal Growth Factor Receptor) CCG TGG TCA TGC TCC (SEQ ID. No 10) (prepared by Hybridon, Inc.)

PEG-CerC14 is PEG(mw2000)-Ceramide with 14 carbon acyl chain.

PEG-CerC20 is PEG(mw2000)-Ceramide with 20 carbon acyl chain.

PEG-DSPE is PEG(mw2000)- 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine

ATTA8-DSPE is N-(ω-N'-acetoxy-octa(14'amino-3',6',9',12'-tetraoxatetradecanoyl))-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (molec weight about 2660). Synthesis of ATTA8-DSPE is fully disclosed in US Provisional Pat. Application Serial No. 60/073,852, filed 23 - Dec – 1997 and US Provisional Pat. Application filed 2-Feb-1998 (Attorney Docket No.: TT&C 16303-005810) both assigned to the assignee of the instant invention and incorporated herein by reference.

Each formulation contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense, as described in Example 4.4, above. All samples were prepared in 300 mM citrate pH 4.0 containing 40% ethanol and extruded 10X through 100 nm filters. Formulations contained phosphorothioate antisense and lipid or empty lipid alone. Samples were dialyzed in HBS (20 mM Hepes, 145 mM NaCl, pH 7.45) to remove ethanol and citrate. Sample lipid concentrations were adjusted such that the injected lipid dose will be 1.8 μmol/mouse/week (5-10 mg AS per kg mouse/week). Samples were filtered (0.22 μm) prior to injection.

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In this experiment female (20-25g) ICR mice (6-8 weeks old) were divided into 9 groups of 6, plus other control groups. Each group received four injections of the same formulation. All injections were 200 μL intravenous (via the lateral tail vein) at a lipid dose of 120 mg/kg. Mice were dosed every week for 3 weeks (4 injections). At 4 weeks, certain

5   groups (treated with lipid and antisense) were given an injection of empty lipid carriers of varying composition to evaluate whether there is rapid clearance of the carrier in the absence of antisense. Blood (25 μl, pipettor) was collected 1 h post-injection each week for 3 weeks by tail nicks. Mice were weighed each week to estimate blood volume (8.0 ml whole blood/100 g body weight). Blood was placed in a glass scintillation vial containing 200 μl of

10   5% EDTA. Solvable (500 μl) was added and the blood was digested for 3 h at 65°C. Samples were decolorized by the addition of 100 μl 70% hydrogen peroxide. Samples were analyzed for radioactivity by liquid scintillation counting. At the end of 4 weeks, mice were terminated by CO_2 inhalation or cervical dislocation preceded by general anesthesia.

The results of this experiment are shown in **Figure 17.** For all formulations

15   not containing antisense ("empty liposomes") repeat dosages demonstrated circulation times reasonably consistent with the first dosage. However, when antisense is used in the formulation, it was surprisingly found that the acyl chain length of the lipid derivatized to the steric barrier (i.e. ATTA or PEG) moiety demonstrates a profound effect on clearance rates. Repeat dosages of PEG-CerC20, PEG-DSPE and ATTA8-DSPE formulations are rapidly

20   cleared from the circulation compared to the first dosage, whereas the PEG-CerC14 formulation is reasonably consistent with the first dosage.

Similar results are demonstrated in **Figure 18**. The formulations were identical to those of **Figure 17**, with the additional formulation of empty vesicles using the same lipids as formulations 4 and 5.

25   Without intending to be bound by any particular theory of action, it is suggested by these results that lipids like the PEG-CerC14 lipid, a lipid which exchanges out of the liposome membrane with a T1/2 on the order of minutes (i.e. 1-60mins) in blood provides a tremendous benefit over lipids like PEG-CerC20, PEG-DSPE and ATTA8-DSPE which do not exchange out, where repeat dosing of a lipid-formulated compound, such as a

30   therapeutic compound or diagnostic compound, is required. The mammalian blood clearance response may not recognize these as foreign antigens if the derivatized lipid is removed

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expeditiously from the liposome surface when in circulation. However, when the derivatized-lipid remains with the formulation for extended periods, a clearance response is invoked, which causes rapid clearance upon repeat dosing. This data suggests that any lipid derivatized with a steric barrier molecule that exchanges out of the liposome membrane faster

5   than PEG-CerC20, PEG-DSPE or ATTA8-DSPE will be superior for use in repeat dosing. For example ATTA8-DMPE, or PEG-CerC8 to C18 all being exchangeable, will have improved circulation characteristics upon repeat administration.

Taken together, it will be evident to one skilled in the art, that on the basis of these teachings, any diagnostic or therapeutic agent that may be delivered in a lipid

10   formulation comprising a steric-barrier derivatized lipid, such as a PEG-lipid or ATTA-lipid, should be tested with both a long and short acyl-chain anchors, in order to determine which formulation is best for repeat dosings.

Further, without intending to be bound by any theory of action, the invention herein may prove to be particularly useful when the bioactive agent being delivered is a non-

15   cytotoxic agent. Cytotoxic agents kill those cells which clear long circulating (i.e. PEG-DSPE) liposomes. This ensures that repeat dosings will not be rapidly cleared, because the cells responsible (usually macrophages) do not survive. In these situations, the acyl-chain length may not be significant. However, where the bioactive agent is non-cytotoxic, such as in the case of antisense drugs (regardless of chemistry or target), plasmids, proteins, etc., and

20   many conventional drugs, the invention will be useful for repeat dosing.

4.10   *In vivo efficacy of repeat doses of encapsulated phosphorothioate c-myc antisense in an oncology model.*

In vivo efficacy of repeat injections of using formulations of the invention are

25   shown in a mouse tumor system in **Figure 19.** This experiment demonstrated efficacy of the antisense formulated according to the invention in a human oncology model, and showed the importance of PEG-acyl chain length on the efficacy of repeat dosings.

Lipid-antisense particle formulation: Formulations were prepared as described in these Examples.

30

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Formu-lation	DSPC (mol%)	Chol (mol%)	DODAP (mol%)	Steric Barrier Derivatized Lipid (name: mol%)	Antisense (c-myc 2mg/ml)
HBS Buffer					Empty
AS4200 (c-myc)	25	45	25	PEG-CerC14 : 5	LR-3280
AS4204 (c-myc)	25	45	25	PEG-CerC20 : 5	LR-3280
AS4204 (c-myc SCR)	25	45	25	PEG-CerC20 : 5	c-myc SCR
AS4204 (PS-2302)	25	45	25	PEG-CerC20 : 5	PS-2302
AS4204(PS-3082)	25	45	25	PEG-CerC20 : 5	PS-3208
c-myc					LR-3280
c-myc SCR					c-myc SCR
PS-2302					PS-2302
PS-3082					PS-3082
AS4200 (no antisense)	25	45	25	PEG-CerC14 : 5	Empty
AS4204 (no antisense)	25	45	25	PEG-CerC20 : 5	Empty

Antisense used were:

LR-3280:	human c-myc gene (phosphorothioate)	
	AAC GTT GAG GGG CAT	(SEQ ID. No 4)
c-myc SCR:	GAA CGG AGA CGG TTT	(SEQ ID. No 17)
PS-2302	human ICAM-1 (phosphorothioate)	
	GCCCAAGCTGGCATCCGTCA	(SEQ ID. No 2)
PS-3082	murine ICAM-1 (Intracellular Adhesion Molecule-1) (phosphorothioate)	
	TGCATCCCCCAGGCCACCAT	(SEQ ID. No 1)

Formulations were diluted in filtered HBS, pH 7.6 to achieve required antisense dose (i.e. lipid dose decreases as well). Samples were filtered (0.22 μm) prior to injection. External buffer was HBS (20 mM Hepes, 145 mM NaCl, pH 7.6). Free antisense

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was dissolved in HBS and adjusted to the required dose by A260 (Extinction coefficients: active and control c-myc = 30.6, PS-2302 = 32.8, PS-3082 = 33.6).

Tumour Inoculum: B16/BL6 murine melanoma cells were maintained in culture in MEM media supplemented with 10% FBS. On day 0 of the study, 3×10^5 cells were injected sub-
5   cutaneously (s.c.) into the dorsal flank (injection volume: 50 μl) of female C57BL/6 mice (20-23 g). Typically, 15% extra mice will be injected so non-spheroidal tumours or mice in which no tumours are observed can be excluded from the study. Tumours were allowed to grow for a period of 5-7 days until tumors reached 50-100 mm^3 prior to initiating treatments with test samples/controls.

10   **Treatment:** On the day of first treatment mice with acceptable tumours were randomly grouped with 5 animals per group. Treatment began when tumours were 50-100 mm^3. Mice were dosed every other day for a total of 7 doses. Administrations were via intravenous tail vein injections (200 ul). Initial drug:lipid ratio of formulation was 0.20 (w/w) and the final drug:lipid ratio (0.14) was held constant; consequently, the lipid concentration varied as
15   samples were diluted to the desired antisense concentration. The antisense dose was 10 mg/kg.

Endpoints: Primary tumour volume was measured using calipers. Length (mm) and width (mm) measurements were made every other day (on non-injection days) for the duration of the study. Tumour height measurements (mm) were made when feasible. Tumour volumes
20   were calculated using the following formulas:

#1   Tumour Volume (mm^3) = (L x W^2)/2

#2   Tumour Volume (mm^3) = (L x W x H) x π/6

Mice were euthanized when tumour volumes reach 10% of body weight or on the first signs of ulceration. Mouse weights were recorded every day during the dosing portion of the study.
25   On termination, all tumours were excised, weighed, observed by FACS analysis or by Northern/Western analysis. Mice were euthanized by CO_2 inhalation or cervical dislocation preceded by general anesthesia.

Results: Figure 9 shows weights of tumors excised and weighed at day 18 for all groups treated with antisense at 10 mg/kg/dose compared with empty lipid controls. Tumour sizes
30   for the AS4200(c-myc) group exhibited the best efficacy and were very consistent with only small ranges in tumour volumes observed (285-451 mm^3). The group treated with free c-myc

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also resulted in smaller tumours but exhibited more variability in tumour volume (156-838 mm³). The encapsulated c-myc controls (c-myc SCR/PS-2302/PS-3082), AS4204(c-myc), empty lipid carriers, and free antisense controls, however, showed no inhibitory effect on tumor volumes over the 18 days when compared to HBS controls.

5   c-myc expression in tumor tissue was also evaluated by FACS. A correlation between tumour size and c-myc protein expression was detected (data not shown).

To determine the importance of the stability of the PEG-polymers, PEG-acyl chain length was evaluated using formulations containing PEG-CerC14 and PEG-CerC20. Interestingly, the formulation containing the PEG-CerC20 (AS4204) showed no apparent

10   efficacy at any of the doses studied. The PEG-CerC14 formulation (AS4200) showed a dose response. The difference observed between the PEG-CerC14 and PEG-CerC20 formulations may reflect the rapid clearance phenomenon that has been observed in other models.

To establish the tolerability of free and encapsulated antisense, mouse weights were measured on a daily basis during the treatment phase of the study. No significant

15   changes in mouse weights for either free or encapsulated formulations were apparent over the course of the dosing phase or throughout the study.

EXAMPLE 5

This example illustrates a high efficiency formulation according to Example 2,

20   but instead of phosphorothioate antisense, employing 1) a phosphodiester antisense compound having exclusively phosphodiester internucleotide linkages (PO-2302 anti-human ICAM-1 GCCCAAGCTGGCATCCGTCA (SEQ ID. No 1)) prepared by Inex Pharmaceuticals (USA), Inc., Hayward CA) or 2) ribozyme molecule to VEGF-R-1 (human Vascular Endothelial Growth Factor Receptor 1) comprising a modified RNA sequence of

25   GAG UUG CUG AUG AGG CCG AAA GGC CGA AAG UCU G (SEQ ID. No 16).

A 15mer of [³H]-phosphodiester antisense oligodeoxynucleotide (PO-2302) in citrate buffer, pH 3.80 (experiments ranged from 10-1000 mM citrate) was mixed with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The final ethanol

30   concentration in the 1 ml preparation was 38% vol/vol. The sample was extruded ten times through three 100 nm filters as described in "Materials and Methods". The sample was

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dialyzed for 2-3 hours in citrate buffer, pH 3.80 (same molarity as experiment), to remove a majority of the excess ethanol. The samples were switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. Non-encapsulated antisense was removed either by this regular dialysis, tangential

5   flow dialysis, or chromatography. Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

Figure 15 illustrates results. Encapsulation efficiency was over 50% across the 10-50 mM citrate range, and all final (administration ready) drug:lipid ratios were greater

10   than 10% by weight. Parallel experiments varying citrate concentration were conducted with phosphorothioate antisense PS-2302. Results are also above 50% encapsulation, and in fact show a higher encapsulation efficiency than phosphodiesters, particularly at higher citrate concentrations.

This experiment was repeated using 20mM citrate instead of 300 mM citrate

15   to encapsulate the ribozyme molecule to VEGF-R-1 (human Vascular Endothelial Growth Factor Receptor 1) GAG UUG CUG AUG AGG CCG AAA GGC CGA AAG UCU G (SEQ ID. No 16). **Figure 20** shows the encapsulation efficiency of the ribozyme at was over 50%, approximately the same as the phosphodiester.

20   **EXAMPLE 6**

This example illustrates a high efficiency formulation as in Example 5, but replacing DODAP with an alternative protonatable lipid. Typically, the preparation for the alternative will be X:DSPC:CHOL:PEG-CerC14 at 20:25:45:10 molar ratio where X can be DODAC, OA, DODMA or any other lipid suitable for the invention.

25   Materials: distearoylphosphatidylcholine, DSPC; cholesterol, CHOL (both from Northern Lipids, Vancouver, BC); N,N-dioleyl-N,N-dimethylammonium chloride, DODAC; Oleylamine, OA (prepared by Steve Ansell, Inex); N-(1-(2,3-Dioleoyloxy) propyl)-N,N,-dimethyl ammonium chloride, DODMA(Avanti Polar Lipids, Alabaster AB, chloride salt prepared by Steve Ansell, INEX); poly(ethylene glycol)2000 coupled to a

30   ceramide derivative with 14 carbon acyl chains, PEG-CerC14 (Zhou Wang, INEX Pharmaceuticals); 13 x 100 mm glass tube; filter sterilized 300 mM citrate buffer, pH 3.9 -

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4.0 (use a 0.2 µm filter). Fully thioated c-myc antisense (INEX (USA), Hayward Ca),

Anhydrous Ethanol (Commercial Alcohols, Toronto, On), Citric acid, Monobasic Sodium

phosphate, Dibasic Sodium phosphate, Sodium hydroxide, HEPES (BDH, Mississauga On).

Deionized water, Chloroform, Methanol, Oligreen™ oligonucleotide reagent (Molecular

5   Probes, Eugene Or), Sodium chloride, Triton X-100, alcohol dehydrogenase reagent kit,

(Sigma Chemical Co., St Louis Mo.),

Lipid stock solutions were made in 100 % ethanol with the working

concentrations of the lipids which is as follows:

DSPC, 20 mg/ml; CHOL, 20 mg/ml (not very soluble above this concentration);

10   DODMA, 20 mg/ml; PEG-CerC14; 50 mg/ml.

To prepare stock solutions of antisense, the antisense molecules were

dissolved in the filtered 300 mM citrate buffer at a concentration of 3.33 mg/ml. Lipids were

mixed in the desired proportions in a 13 x 100 mm glass tube to achieve a final volume of 0.4

ml of lipids using 100 % ethanol as listed in table 1, below:

15

Table 1. Proportional mixture of lipids in a 13 x 100 mm glass test tube.

Lipid	Mol %	M. Wt.	mg	µmol	Stock (mg/ml)	Vol of Stock (µl)
DODMA	20	652.6	1.69	2.60	20	84.5
DSPC	25	790	2.57	3.25	20	115
CHOL	45	386.7	2.26	5.85	20	113.1
PEG-CerC14	10	2600	3.38	1.30	50	67.6
	100		9.9	13.00		380.2

25   In a separate 13 x 100 mm glass tube, 0.6 ml of antisense at 3.33 mg/ml was added. The

pH of this solution should be 3.9 - 4.0. (*NOTE:* the antisense concentration is NOT determined

by weight but rather by measuring absorbance at 260 nm). The lipid mixture solution was

warmed to 65°C for about 2 minutes. The antisense tube was vortexed and during this time,

using a Pasteur pipette, the lipids (in ethanol) were added slowly in a dropwise manner. The

30   mixture will get "cloudy" and some bubbles may be observed due to the ethanol, but no

aggregates should be present. The resulting volume of the antisense-lipid mixture was 1.0 ml

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with a 10 mg (13 umol) total lipid at 13 μmols, 2 mg of antisense, and 38 % ethanol, vol/vol.
It can be expected that the pH to rise to about 4.4.

        The antisense-lipid mixture was subjected to 5 (five) cycles of freezing in liquid
nitrogen and thawing at 65°C in a waterbath. After each thaw, the mixture was vortexed briefly.

5   Subsequently, the mixture was passed 10 times through three stacked 100 nm polytcarbonate
filters (Poretics) or extruded using a pressurized extruder apparatus with a thermobarrel
attachment (Lipex Biomembranes). The temperature and nitrogen pressure during extrusion
were 65°C and no more than 200 psi to 300 psi, respectively. Each pass should take no more
than 2 minutes and is vortexed after each pass.

10   After extrusion, the mixture was dialyzed in a dialysis tubing (3500 Mwt cutoff;
SpectraPor) for 1 hour in 300 mM citrate at pH 3.9-4.0, removing the ethanol. The mixture was
transferred into 5 L of HBS buffer at pH 7.5 and allowed to further dialyze to a minimum of 12
hours, to neutralize the DODMA and release any surface bound antisense associated with the
vesicles. Alternatively, tangential flow dialysis, ion exchange-chromatography or gel filtration

15   chromatography can be used to process the extruded antisense-lipid mixture to an administration
ready preparation.

EXAMPLE 7

        This example illustrates a high efficiency formulation as in Example 5, but

20   replacing DSPC with SM to generate a preparation of DODAP:SM:CHOL:PEG-CerC14 at
20:25:45:10 molar ratio.   Antisense is processed with the formulation for a standard 1.0 ml
volume, which can be scaled up proportionately as required.

        Materials: Sphingomyelin SM; cholesterol, CHOL; dimethylaminopropane,
DODAP; polyethylene glycol coupled to a ceramide derivative with 14 carbon acyl chains,

25   PEG-CerC14; 13 x 100 mm glass tube; filter sterilized 300 mM citrate buffer, pH 3.9 - 4.0
(use a 0.2 μm filter).

        Lipid stock solutions were made in 100 % ethanol with the working
concentrations of the lipids which is as follows:

        SM, 20 mg/ml; CHOL, 20 mg/ml (not very soluble above this concentration); DODAP,

30   20 mg/ml; PEG-CerC14; 50 mg/ml.

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To prepare stock solutions of antisense, the antisense molecules were dissolved in the filtered 300 mM citrate buffer at a concentration of 3.33 mg/ml. Lipids were mixed in the desired proportions in a 13 x 100 mm glass tube to achieve a final volume of 0.4 ml of lipids using 100 % ethanol as listed in Table 2, below:

5   **Table 2. Proportional mixture of lipids in a 13 x 100 mm glass test tube.**

Lipid	Mol %	M. Wt.	mg	µmol	Stock (mg/ml)	Vol of Stock (µl)
DODAP	20	684.5	1.78	2.60	20	89.0
SM	25	703	2.30	3.27	20	115
CHOL	45	386.7	2.26	5.85	20	113.1
PEG-CerC14	10	2600	3.38	1.30	50	67.6
	100		9.72	13.02		384.7

In a separate 13 x 100 mm glass tube, 0.6 ml of antisense at 3.33 mg/ml was added. The
15   pH of this solution should be 3.9 - 4.0. (*NOTE:* the antisense concentration is NOT determined by weight but rather by measuring absorbance at 260nm). The lipid mixture solution was warmed to 65°C for about 2 minutes. The antisense tube was vortexed and during this time, using a Pasteur pipette, the lipids (in ethanol) were added slowly in a dropwise manner. The mixture will get "cloudy" and some bubbles may be observed due to the ethanol, but no
20   aggregates should be present. The resulting volume of the antisense-lipid mixture was 1.0 ml with a 10 mg (13 umol) total lipid at 13 µmols, 2 mg of antisense, and 38 % ethanol, vol/vol. It can be expected that the pH to rise to about 4.4.

The antisense-lipid mixture was subjected to 5 (five) cycles of freezing in liquid nitrogen and thawing at 65°C in a waterbath. After each thaw, the mixture was vortexed briefly.
25   Subsequently, the mixture was passed 10 times through three stacked 100 nm polytcarbonate filters (Poretics) or extruded using a pressurized extruder apparatus with a thermobarrel attachment (Lipex Biomembranes). The temperature and nitrogen pressure during extrusion were 65°C and no more than 200 psi to 300 psi, respectively. Each pass should take no more than 2 minutes and is vortexed after each pass.

30   After extrusion, the mixture was dialyzed in a dialysis tubing (3500 Mwt cutoff; SpectraPor) for 1 hour in 300 mM citrate at pH 3.9-4.0, removing the ethanol. The mixture was transferred into 5 L of HBS buffer at pH 7.5 and allowed to further dialyze to a minimum of 12

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hours, to neutralize the DODAP and release any surface bound antisense associated with the vesicles. Alternatively, tangential flow dialysis, ion exchange-chromatography or gel filtration chromatography can be used to process the extruded antisense-lipid mixture to an administration ready preparation.

EXAMPLE 8

This example illustrates a high efficiency formulation as in Example 5, but replacing PEG-CerC14 with ATTA8-DSPE to prepare DODAP:DSPC:CHOL:ATTA8-DSPE at 40:10:45:5 molar ratio of antisense formulation.

Materials: distearoylphosphatidylcholine, DSPC; cholesterol, CHOL; dimethylaminopropane, DODAP; N-(ω-N'-acetoxy-octa(14'amino-3',6',9',12'-tetraoxatetradecanoyl))-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, ATTA8-DSPE; 13 x 100 mm glass tube; filter sterilized 300 mM citrate buffer, pH 3.9 - 4.0 (use a 0.2 μm filter).

Lipid stock solutions were made in 100 % ethanol with the working concentrations of the lipids which is as follows:

DSPC, 20 mg/ml; CHOL, 20 mg/ml (not very soluble above this concentration); DODAP, 20 mg/ml; ATTA8-DSPE; 50 mg/ml.

To prepare stock solutions of antisense, the antisense molecules were dissolved in the filtered 300 mM citrate buffer at a concentration of 3.33 mg/ml. Lipids were mixed in the desired proportions in a 13 x 100 mm glass tube to achieve a final volume of 0.4 ml of lipids using 100 % ethanol as listed in Table 3, below:

Table 3. Proportional mixture of lipids in a 13 x 100 mm glass test tube.

Lipid	Mol %	M. Wt.	mg	μmol	Stock (mg/ml)	Vol of Stock (μl)
DODAP	40	684.5	4.16	6.08	20	208
DSPC	10	790	1.2	1.52	20	60
CHOL	45	386.7	2.6	6.72	20	130
ATTA8-DSPE	5	2638	2.0	0.76	50	40
	100		10.26	15.1		438

In a separate 13 x 100 mm glass tube, 0.6 ml of antisense at 3.33 mg/ml was added. The pH of this solution should be 3.9 - 4.0. (*NOTE:* the antisense concentration is <u>NOT</u> determined

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by weight but rather by measuring the absorbance at 260nm). The lipid mixture solution was warmed to 65°C for about 2 minutes. The antisense tube was vortexed and during this time, using a Pasteur pipette, the lipids (in ethanol) were added slowly in a dropwise manner. The mixture will get "cloudy" and some bubbles may be observed due to the ethanol, but no

5   aggregates should be present. The resulting volume of the antisense-lipid mixture was 1.0 ml with a 10 mg (13 umol) total lipid at 13 μmols, 2 mg of antisense, and 38 % ethanol, vol/vol. It can be expected that the pH to rise to about 4.4.

The antisense-lipid mixture was subjected to 5 (five) cycles of freezing in liquid nitrogen and thawing at 65°C in a waterbath. After each thaw, the mixture was vortexed briefly.

10   Subsequently, the mixture was passed 10 times through three stacked 100 nm polytcarbonate filters (Poretics) or extruded using a pressurized extruder apparatus with a thermobarrel attachment (Lipex Biomembranes). The temperature and nitrogen pressure during extrusion were 65°C and no more than 200 psi to 300 psi, respectively. Each pass should take no more than 2 minutes and is vortexed after each pass.

15   After extrusion, the mixture was dialyzed in a dialysis tubing (3500 Mwt cutoff; SpectraPor) for 1 hour in 300 mM citrate at pH 3.9-4.0, removing the ethanol. The mixture was transferred into 5 L of HBS buffer at pH 7.5 and allowed to further dialyze to a minimum of 12 hours, to neutralize the DODAP and release any surface bound antisense associated with the vesicles. Alternatively, tangential flow dialysis, ion exchange-chromatography or gel filtration

20   chromatography can be used to process the extruded antisense-lipid mixture to an administration ready preparation.

EXAMPLE 9

This example illustrates use of tangential flow dialysis to clean up a large scale

25   (>50 ml) preparation of extruded antisense-lipid mixture to obtain an administration ready preparation. Tangential Flow Diafiltration has been shown to be useful in four functions in the formulation process 1) buffer exchange, 2) removal of ethanol, 3) removal of unencapsulated antisense and 4) concentration of the formulation. Using TF it is demonstrated that it is possible to efficiently exchange these components using only 10-15 sample volumes with a single buffer

30   system at a very significant reduction in the process time.

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Materials for Tangential Flow Dialysis: Microcross Sampler™ Tangential Flow column (Microgon, Laguna Hills, Ca) Masterflex™ console drive and Easyload™ Pump head (Cole-Parmer, Vernon Hills Ill.), Extruder (Lipex Biomembranes, Vancouver BC), Polycarbonate membranes, 100 μm, (AMD Manufacturing, Mississauga On).

5   Antisense (c-myc) is prepared by dissolving in 300 mM Na Citrate buffer to a final concentration of 4.17 mg/ml for c-myc as verified by absorbance at 260 nm. The antisense stock solution is typically warmed to 65°C for 2 minutes to dissolve and to remove secondary structure. AS4200 consists of DODAP:DSPC:CHOL:PEG-CER-14 at the percent mol ratio of 25:20:45:10 and the lipids are aliquoted from stock solutions to a total concentration of 10

10   mg/0.400 ml in anhydrous ethanol. In this study 50 - 60 ml scale formulations were produced. Thus 20-24 ml of the ethanolic lipid solution is added dropwise, at room temperature, using a peristaltic pump at 1ml/nin into 30 - 36 ml of the AS solution which is stirring in a 100 ml round bottom flask with a 2 cm magnet stir bar (Stirrer setting 2-3). After mixing, the lipid/antisense suspension was pipetted into a 100 ml extruder prepared with 2-3, 100 μm polycarbonate

15   membranes and pre-equilibrated at 65°C. The suspension was extruded using ten passes at ~300 psi. After extrusion the formulation was processed using tangential flow diafiltration.

Tangential Flow Ultrafiltration. A 230 cm^2 Microcross tangential flow cartridge (50 kDa cut off) was attached to a Masterflex peristaltic pump, sample reservoir and buffer reservoir using Tygon tubing. The tubing length was adjusted so that the total circuit of

20   tubing, pump and TF cartridge had a total dead volume of 30 ml. To this system a 60 ml sample reservoir was attached. The sample was loaded into the tubing and reservoir by running the peristaltic pump at a low speed. After loading, the system was closed and the pump speed gradually increased to the pump maximum (approx. 100 ml/min) until the initial TF cartridge inlet pressure was 12-15 psi and the outlet pressure was 8-11 psi. When the system pressure

25   stabilized, both the filtrate outlet and the buffer reservoir were opened. Opening these valves allowed filtrate to flow out of the cartridge at ~ 10-15 ml/min while wash buffer (i.e. PBS, pH 7.5) was being collected. For a 50 - 60 ml formulation 700 - 900 ml of buffer was used to "wash" the sample. Fractions (10 ml) of the filtrate were collected for analysis of ethanol removal, pH, and antisense. After diafiltration was completed the wash buffer reservoir was closed and with

30   the pump continuing to run, filtrate was allowed to flow, concentrating the sample, typically reducing the preparation volume to the tubing dead volume (30 - 35 ml). The sample was

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collected from the system and the tubing and column were washed with 15 ml wash buffer to remove any remaining formulation.

Antisense Quantification. Antisense concentration was normally determined by measuring absorbance at 260nm as outlined in the current protocol. Briefly, antisense stock
5   solutions were quantified by diluting 1:500 in MilliQ water and measuring absorbance. TF filtrate fractions were diluted 1:10 in MilliQ water and absorbance was measured. Antisense in suspension with lipids was measured by adding 10 µl of the suspension to 250 µl MilliQ water. A monophase was created by adding 750 µl $CHCl_3$/MeOH (2.1:1) and 100 µl MeOH. Immediately after vortexing the mixture the absorbance was measured at 260 nm. In each case
10   the extinction coefficient for the given antisense was multiplied by the dilution factor to determine the antisense concentration.

Lipid Quantification. As outlined in the current protocol, 50 µl aliquots of the lipid/antisense suspension was diluted with 100 µl MilliQ water and submitted for analysis by HPLC. The percent **encapsulation efficiency** of the formulation is determined by dividing the
15   Drug/Lipid ratio of the finished product by the initial Drug/Lipid ratio formed when the lipid and antisense stock solutions are mixed.

Ethanol Assay. Ethanol in the TF filtrate was determined using an alcohol dehydrogenase reagent kit supplied by Sigma Chemical Co.

DEAE Sephadex chromatography. A suspension of the processed formulation
20   was loaded onto a 1 X 10 cm column of DEAE sephadex equilibrated in 20 mM PBS, pH 7.5. After eluting through the column the formulation was collected into a sterile falcon tube. The volume, antisense and lipid concentration were measured to determine recovery.

Particle Size. The particle size of the formulation was measured by QELS using a Nicomp Particle sizer, (Nicomp, Santa Barbara, CA.) and particle sizes are reported in the
25   particle mode with volume weighing.

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Results of Large Scale Preparations:

Assay	Initial Lipid Content (mg/ml)	Initial Antisense Content (mg/ml)	Final Lipid Content (mg/ml)	Final Antisense Content (mg/ml)	Initial Drug:Lipid	Final Drug:Lipid	Encaps. Effic.
A	10.581	1.936	14.604	1.681	0.183	0.115	63%
B	8.727	2.284	7.926	1.008	0.262	0.127	48%
C	11.06	2.97	2.69	0.556	0.286	0.207	77%

EXAMPLE 10

Phosphodiester and phosphorothioate antisense oligonucleotides encapsulated according to the methods in Example 2 and 5-9 were examined for their relative susceptibility to nuclease digestion by serum or S1 nuclease. Protection of the phosphodiester-linked oligonucleotide was significantly higher in serum when encapsulated as opposed to the free, raising the $T_{1/2}$ of degradation from 10 mins to at least 8h. Free phosphorothioate oligodeoxynucleotide showed significant breakdown in serum within 30 minutes, however encapsulated phosphorothioate oligodeoxynucleotide did not show any sign of degradation even after 24h incubation in serum. *In vivo* data agrees with these findings, showing no sign of degradation of the encapsulated phosphorothioate antisense until 8h.

As a positive control, the free phosphodiester and phosphorothioate antisense were subjected to very potent levels of S1 nuclease ($100U/50\mu g$) (1U of S1 nuclease will digest 1 ug DNA per minute at 37°C). The enzyme completely digested the free phosphodiester and phosphorothioate within seconds after its addition. The encapsulated phosphodiester under the same conditions was over 90% intact at 24h, and the encapsulated phosphorothioate was fully intact at 24h.

The experiments were conducted as described in the specification, or modified as follows.

S1 Nuclease Digestion. $50\mu g$ aliquots containing free, encapsulated, or encapsulated + 0.5% Triton X100 were aliquoted into 1.5 ml eppendorf tubes. To the tubes were added 10 μl 10X S1 nuclease buffer, dH2O (to make final volume 100 μl), and, just prior to digestion, 100U of S1 nuclease to each eppendorf tube. The tubes were sealed with parafilm and incubated at 55°C. A sample of the free, encapsulated, or encapsulated + 0.5% Triton X100 not digested by nuclease (standard) was frozen in liquid nitrogen in an eppendorf

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tube and stored at -20°C. At each desired time point, an aliquot of each sample was collected, added to GDP buffer containing proteinase K (133 μg/ml) and immediately frozen in liquid nitrogen in order to stop the reaction. Once all of the time points were collected, the samples were incubated at 55°C in a waterbath to activate proteinase K enabling it to

5   denature any remaining S1 nuclease. Proteinase K digested samples were applied to polyacrylamide gels, described below, to assess levels of S1 nuclease degradation

   Normal Murine/Human Serum Digestion. 50μg of the free, encapsulated, or encapsulated + 0.5% Triton X100 was aliquoted into 1.5 ml eppendorf tubes. To the tubes we added 45 μl normal murine/human serum, dH2O (to make final volume 50 μl), to each

10   eppendorf tube. The tubes were sealed with parafilm and incubated at 37°C. A sample of the free, encapsulated, or encapsulated + 0.5% Triton X100 not digested by nuclease (standard) was frozen in liquid nitrogen in an eppendorf tube and stored at -20°C. Aliquots were taken at various time points, added to GDP buffer containing proteinase K (133 μg/ml) and immediately frozen in liquid nitrogen to stop the reaction. Once all of the time points were

15   collected, the samples were incubated at 55°C in a waterbath to activate proteinase K enabling it to denature any remaining exonuclease. Proteinase K digested samples were applied to polyacrylamide gels to assess levels of exonuclease degradation
   Micrococcal Nuclease. An alternative standard nuclease assay not employed in the present experiment is the assay disclosed by Rahman et al. US Pat. 5665710, wherein nucleic acid/

20   lipid particles are incubated for 30 mins at 37°C in presence of an excess of micrococcal nuclease in 1 mM CaCl$_2$.

   Polyacrylamide Gel Electrophoresis (PAGE). Prepared 14 cm X 16 cm X 7.5mm polyacrylamide (15% or 20%) gels in 7M urea and TBE. Approximately 300 ng of sample (at each time point) and standard were aliquoted into eppendorf tubes. An equivalent

25   volume of 2X loading buffer was added to each sample. The samples were then heated in a waterbath to 90°C for 3 min to reduce secondary structures and then applied to the gel. The loaded gel was electrophoresed at 600V for 10 min (to sharpen the band) and then at 300V for the duration of the gel. The gel was incubated in 1X SyberGreen I stain in TBE for a minimum of 15 min and then photographed while illuminated under UV light (3.5 sec

30   exposure, 4.5 aperture).

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VII.   Conclusion

As discussed above, the present invention provides methods of preparing lipid-encapsulated therapeutic agent (nucleic acid) compositions in which the therapeutic agent (nucleic acid) portion is encapsulated in large unilamellar vesicles at a very high efficiency.

5   Additionally, the invention provides compositions prepared by the method, as well as methods of introducing therapeutic agents (nucleic acids) into cells. The compositions are surprisingly efficient in transfecting cells, both *in vivo* and *in vitro*.

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as

10   if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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      (D) PROVINCE: B.C.
      (E) COUNTRY: Canada
      (F) POSTAL CODE: V5J 5J8
      (G) TELEPHONE:
      (H) TELEFAX:
      (I) TELEX:

   (ii) TITLE OF INVENTION: High Efficiency Encapsulation of Charged
       Therapeutic Agents in Lipid Vesicles

   (iii) NUMBER OF SEQUENCES: 17

   (iv) CORRESPONDENCE ADDRESS:
      (A) ADDRESSEE: Smart & Biggar
      (B) STREET: Box 11560, Vancouver Centre
             2200 - 650 West Georgia Street
      (C) CITY: Vancouver
      (D) PROVINCE: B.C.
      (E) COUNTRY: Canada
      (F) POSTAL CODE: V6B 4N8

   (v) COMPUTER READABLE FORM:
      (A) MEDIUM TYPE: Floppy disk
      (B) COMPUTER: IBM PC compatible
      (C) OPERATING SYSTEM: PC-DOS/MS-DOS
      (D) SOFTWARE: Word Perfect

   (vi) CURRENT APPLICATION DATA:
      (A) APPLICATION NUMBER:
      (B) FILING DATE:
      (C) CLASSIFICATION:

   (vii) PRIOR APPLICATION DATA:
      (A) APPLICATION NUMBER: US 08/856,374
      (B) FILING DATE: 14-MAY-1997

   (viii) ATTORNEY/AGENT INFORMATION:
      (A) NAME: Smart & Biggar
      (C) REFERENCE/DOCKET NUMBER: 80472-2

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(2) INFORMATION FOR SEQ ID NO:1:

JA001320
GENV-00011188

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```
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 20
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid

      (iii) HYPOTHETICAL: no

       (iv) ANTI-SENSE: yes

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

  TGCATCCCCC AGGCCACCAT                                              20


(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 20
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid

      (iii) HYPOTHETICAL: no

       (iv) ANTI-SENSE: yes

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

  GCCCAAGCTG GCATCCGTCA                                              20


(2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
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            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid

      (iii) HYPOTHETICAL: no

       (iv) ANTI-SENSE: yes

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

   GGTGCTCACT GCGGC                                                  15
```

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(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 15
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

    (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AACGTTGAGG GGCAT   15

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 16
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

    (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TAACGTTGAG GGGCAT   16

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 24
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

    (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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TATGCTGTGC CGGGGTCTTC GGGC   24

(2) INFORMATION FOR SEQ ID NO:7:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 18
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid

      (iii) HYPOTHETICAL: no

       (iv) ANTI-SENSE: yes

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTGCCGGGGT CTTCGGGC   18

(2) INFORMATION FOR SEQ ID NO:8:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 18
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid

      (iii) HYPOTHETICAL: no

       (iv) ANTI-SENSE: yes

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGACCCTCCT CCGGAGCC   18

(2) INFORMATION FOR SEQ ID NO:9:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 18
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: other nucleic acid

      (iii) HYPOTHETICAL: no

       (iv) ANTI-SENSE: yes

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        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

    TCCTCCGGAG CCAGACTT   18

(2) INFORMATION FOR SEQ ID NO:10:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 15
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid

       (iii) HYPOTHETICAL: no

        (iv) ANTI-SENSE: yes

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

    CCGTGGTCAT GCTCC   15

(2) INFORMATION FOR SEQ ID NO:11:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 21
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid

       (iii) HYPOTHETICAL: no

        (iv) ANTI-SENSE: yes

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

    CAGCCTGGCT CACCGCCTTG G   21

(2) INFORMATION FOR SEQ ID NO:12:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 20
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid

       (iii) HYPOTHETICAL: no

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(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CAGCCATGGT TCCCCCCAAC   20

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

    (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTTCTCGCTG GTGAGTTTCA   20

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

    (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TCTCCCAGCG TGCGCCAT   18

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

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    (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTGCTCCATT GATGC   15

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 34
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

    (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GAGUUGCUGA UGAGGCCGAA AGGCCGAAAG UCUG   34

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 15
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid

    (iii) HYPOTHETICAL: no

    (iv) ANTI-SENSE: yes

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAACGGAGAC GGTTT   15

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CLAIMS

1.   A method for preparation of a composition comprising lipid-encapsulated therapeutic agent particles, said method comprising the steps of:

5   (a)   combining a mixture of lipids comprising at least a first lipid component and a second lipid component with a buffered aqueous solution of a charged therapeutic agent to form an intermediate mixture containing lipid-encapsulated therapeutic agent particles, said first lipid component being selected from among lipids containing a protonatable or deprotonatable group that has a pKa such that the lipid is in a charged form at

10   a first pH and a neutral form at a second pH, preferably near physiological pH, said buffered solution having a pH such that the first lipid component is in its charged form when in the buffered solution, said first lipid component being further selected such that the charged form is cationic when the charged therapeutic agent is anionic in the buffered solution, and anionic when the charged therapeutic agent is cationic in the buffered solution, and said second lipid

15   component being selected from among lipids that prevent particle aggregation during lipid-therapeutic agent particle formation, and

                (b) changing the pH of the intermediate mixture to neutralize at least some exterior surface charges on said lipid-encapsulated therapeutic agent particles to provide at least partially-surface neutralized lipid-encapsulated therapeutic agent particles.

20

2.   The method of claim 1, wherein the therapeutic agent is a polyanionic nucleic acid.

3.   The method of claim 2, wherein said nucleic acid is an antisense

25   nucleic acid.

4.   The method of claim 3, wherein said antisense nucleic acid contains linkages selected from the group consisting of phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate, phosphoroselenate and amidate linkages.

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5.   The method of any of claims 2 to 4, wherein said nucleic acid is an antisense nucleic acid.

6.   The method of any of claims 2 to 5, wherein said nucleic acid contains

5   exclusively phosphodiester linkages.

7.   The method of claim 6, wherein the buffered solution comprises 10 to 50 mM citrate or phosphate buffer.

10   8.   The method of any of claims 2 to 5, wherein the nucleic acid contains at least some phosphorothioate or phosphordithioate linkages.

9.   The method of claim 8, wherein the buffered solution comprises 10 to 300 mM citrate or phosphate buffer.

15

10.   The method of claim 2, wherein said nucleic acid is a ribozyme.

11.   The method of any of claims 2 to 10, wherein said composition consists essentially of lipid-nucleic acid particles, said particles having a size of from 70 nm

20   to about 200 nm.

12.   The method of any of claims 1 to 11, wherein said mixture of lipids in step (a) is a mixture of lipids in alcohol.

25   13.   The method of any of claims 1 to 12, wherein the first lipid component is an amino lipid.

14.   The method of any of claims 1 to 13, wherein the second lipid component is a polyethylene glycol-modified or polyamide oligomer-modified lipid.

30

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15.   The method of any of claims 1 to 14, wherein said lipids present in said lipid mixture comprises an amino lipid having a pKa of from about 5 to about 11, a neutral lipid, Chol and a PEG-modified or Polyamide oligomer-modified lipid.

5   16.   The method of claim 15, wherein said lipids are present in molar percents of about 25-45% neutral lipid, 35-55% Chol, 10-40% amino lipid and 0.5-15% PEG-modified or polyamide oligomer-modified lipid.

17.   The method of any of claims 1 to 16, wherein the second lipid

10   component is a PEG-Ceramide.

18.   The method of claim 17, wherein said mixture of lipids comprises DODAP, DSPC, Chol and PEG-Cer14.

15   19.   The method of claim 18, wherein said lipids are present in molar percents of about 25-45% DSPC, 35-55% Chol, 10-40% DODAP and 0.5-15% PEG-Cer14.

20.   The method of claim 17, wherein said mixture of lipids comprises DODAP, POPC, Chol and PEG-Cer14.

20

21.   The method of claim 17, wherein said mixture of lipids comprises DODAP, SM, Chol and PEG-Cer14.

22.   The method of any of claims 1 to 21, wherein the step of changing the

25   pH is performed using tangential flow dialysis.

23.   A composition comprising lipid-therapeutic agent particles comprising a lipid portion and a charged therapeutic agent, said charged therapeutic agent being encapsulated in said lipid portion, wherein said lipid portion comprises at least a first lipid

30   component and a second lipid component, said first lipid component being selected from among lipids containing a protonatable or deprotonatable group that has a pKa such that the

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lipid is in a charged form at a first pH and a neutral form at a second PH, preferably near

physiological pH, and said first lipid component being further selected such that the charged

form is cationic when the therapeutic agent is anionic and anionic when the therapeutic agent

is cationic, and said second lipid component being selected from among lipids that prevent

5   particle aggregation during lipid-nucleic acid particle formation and which exchange out of

the lipid particle at a rate greater than PEG-CerC20.

24.   A composition comprising lipid-therapeutic agent particles comprising

a lipid portion and a charged therapeutic agent, said charged therapeutic agent being

10   encapsulated in said lipid portion, wherein said lipid portion comprises at least a first lipid

component and a second lipid component, said first lipid component being selected from

among lipids containing a protonatable or deprotonatable group that has a pKa such that the

lipid is in a charged form at a first pH and a neutral form at a second pH, preferably near

physiological pH, and said first lipid component being further selected such that the charged

15   form is cationic when the therapeutic agent is anionic and anionic when the therapeutic agent

is cationic, and said second lipid component being selected from among lipids that prevent

particle aggregation during lipid-nucleic acid particle formation, said particles having a

nucleic acid/lipid ratio of at least 10% by weight and a size of from about 70 to about 200

nm.

20

25.   The composition according to claim 23 or 24, wherein at least some of

the protonatable or deprotonatable groups disposed on the exterior surface of the particles

have been neutralized.

25   26.   The composition according to any of claims 23 to 25, wherein the

therapeutic agent is anionic.

27.   The composition according to claim 26, wherein the therapeutic agent

is a polyanionic nucleic acid.

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28.   The composition according to claim 27, wherein the nucleic acid is an antisense nucleic acid.

29.   The composition according to claim 27 or 28, wherein at least 50% of the nucleic acid in the composition is encapsulated within the particle.

5

30.   The composition of claim 29, wherein at least 90% of the nucleic acid in the composition is encapsulated within the particle.

31.   The composition of any of claims 27 to 30, wherein the nucleic acid

10   has exclusively phosphodiester linkages.

32.   The composition of any of claims 27 to 31, wherein at least 50% of the nucleic acid in the composition is encapsulated within the particle.

15   33.   The composition of claim 32, wherein at least 90% of the nucleic acid in the composition is encapsulated within the particle.

34.   The composition of any of claims 27 to 30, wherein said nucleic acid is a ribozyme.

20

35.   The composition of any of claims 23 to 34, wherein the first lipid component is an amino lipid.

36.   The composition of any of claims 23 to 35, wherein the second lipid

25   component is a polyethylene glycol-modified or polyamide oligomer-modified lipid.

37.   The composition of any of claims 23 to 36, wherein the lipid portion comprises a neutral lipid, an amino lipid, cholesterol and PEG-modified or polyamide oligomer-modified lipid, and wherein said lipids are present at molar percents of about 25-

30   45% neutral lipid, 35-55% cholesterol, 10-40% amino lipid and 0.5-15% PEG-modified or polyamide oligomer-modified lipid.

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38.   The composition of claim 37, wherein said lipid portion comprises DODAP, DSPC, Chol and PEG-Cer14.

5

39.   The composition of claim 38, wherein the lipids are present in molar percents of about 25-45% DSPC, 35-55% Chol, 10-40% DODAP and 0.5-15% PEG-Cer14.

40.   The composition of claim 37, wherein said lipid portion comprises DODAP, POPC, Chol and PEG-Cer14.

10

41.   The composition of claim 37, wherein said lipid comprises of DODAP, SM, Chol and PEG-Cer14.

42.   A method for introducing a nucleic acid into a cell, comprising

15   contacting a cell with a lipid-nucleic acid composition prepared according to any of claims 1 to 22 for a period of time sufficient to introduce the nucleic acid into said cell.

43.   A method for the treatment or prevention of a disease characterized by aberrant expression of a gene in a mammalian subject comprising,

20   preparing a lipid-encapsulated therapeutic nucleic acid particle according to the method of any of claims 1 to 22, wherein the therapeutic nucleic acid component hybridizes specifically with the aberrantly expressed gene; and

              administering a therapeutically effective or prophylactic amount of the particle to the mammalian subject, whereby expression of the aberrantly expressed gene is reduced. .

25

44.   The method of claim 43, wherein the gene is selected from among ICAM-1, c-myc, c-myb, ras, raf, erb-B-2, PKC-alpha, IGF-1R, EGFR, VEGF and VEGF-R-1.

45.   The method of claim 43, wherein the disease is a tumor.

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46.   The method of claim 43, wherein the disease is characterized by inflammation.

47.   The method of claim 43, wherein the disease is an infectious disease.

48.   The method of claim 43, wherein the therapeutically effective amount of the particle is administered to the mammalian subject by intravenous injection.

49.   The method of claim 48, wherein the therapeutically effective amount of the particle is administered to the mammalian subject by intravenous injection at an injection site, and wherein the disease is localized at a disease site distal to the injection site.

50.   The method of any of claims 43 to 49, wherein the particles are administered to the mammal in a plurality of doses, at intervals of from one to three weeks.

51.   The method of any of claims 43 to 50, wherein the nucleic acid comprises exclusively phosphodiester linkages.

52.   A method of preventing expression of a disease-associated gene in a mammalian cell comprising,

preparing a lipid-therapeutic oligonucleotide particle according to any of claims 1 to 22 containing an antisense therapeutic agent; and

exposing the mammalian cell to the lipid-therapeutic oligonucleotide particle for a period of time sufficient for the therapeutic oligonucleotide component to enter the cell;

wherein the antisense therapeutic agent has a sequence complementary to the disease-associated gene and reduces the production of the gene product of the disease-associated gene in the cell.

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53.   A pharmaceutical composition comprising lipid-therapeutic agent particles prepared according to any of claims 1 to 22 and a pharmaceutically acceptable carrier.

5

54.   A method for treatment or prevention of a disease characterized by aberrant expression of a gene in a mammalian subject comprising, administering to mammalian subject a composition comprising lipid-encapsulated nucleic acid particles, wherein the lipid- encapsulated nucleic acid particles contain at least 10% by weight of nucleic acids and the nucleic acids have exclusively phosphodiester linkages.

10

55.   A composition comprising lipid-encapsulated nucleic acid particles, wherein the lipid- encapsulated nucleic acid particles contain at least 10% by weight of nucleic acids and the nucleic acids have exclusively phosphodiester linkages.

15

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pH 4.0 INTERNAL

pH 4.0 EXTERNAL

COLUMN CHROMATOGRAPHY

1. EXCHANGE pH 4.0 CITRATE
    FOR pH 7.5 HBS

2. NEUTRALIZE SURFACE
    DODAP; ANTISENSE RELEASE

3. REMOVAL OF NON-
    ENCAPSULATED ANTISENSE

RELEASE OF
SURFACE
ANTISENSE

pH 4.0 INTERNAL

pH 7.5 EXTERNAL

FIG. 1

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DODAP: AL-1

OTHER AMINO LIPIDS:

R^1 AND/OR R^2 ARE H,
ALKYL OR FATTY ALKYL GROUPS
R^3 IS H, LOWER ALKYL.

R^{11} AND/OR R^{12} ARE LOWER ALKYL/LOWER ACYL, FATTY ALKYL,
FATTY ACYL.

(AT LEAST ONE OF R^{11} OR R^{12} IS A LONG CHAIN ALKYL OR ACYL
GROUP)

R^{13} AND R^{14} ARE EACH H, LOWER ALKYL.

FIG. 2A

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FIG. 2B

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FIG. 3

FIG. 4

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FIG. 5

FIG. 6

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IMMEDIATELY AFTER REMOVAL OF FREE ANTISENSE

VOLUME WEIGHTING:
MEAN DIAMETER = 119.3 nm
STD DEVIATION = 32.2 nm (27.0 %)
CUMULATIVE RESULTS:
25 % OF DISTRIBUTION <   88.60 nm
50 % OF DISTRIBUTION < 106.74 nm
75 % OF DISTRIBUTION < 127.93 nm
90 % OF DISTRIBUTION < 151.04 nm
99 % OF DISTRIBUTION < 199.22 nm

AFTER 2 MONTH STORAGE AT 4°C

VOLUME WEIGHTING:
MEAN DIAMETER = 114.2 nm
STD DEVIATION = 27.8 nm (24.3%)
CUMULATIVE RESULTS:
25 % OF DISTRIBUTION <   86.96 nm
50 % OF DISTRIBUTION < 102.86 nm
75 % OF DISTRIBUTION < 121.31 nm
90 % OF DISTRIBUTION < 140.78 nm
99 % OF DISTRIBUTION < 183.74 nm

FIG. 7

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FIG. 8

FIG. 9

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FIG. 10

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FIG. 11

FIG. 12

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FIG. 13

FIG. 14

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FIG. 15

FIG. 16

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FIG. 17

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FIG. 18

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FIG. 19

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FIG. 20

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FIG. 21

FIG. 22

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FIG. 23